INVOLOVEMENT OF OXIDATIVE STRESS IN THE PATHOGENESIS OF SCHIZOPHRENIA: FOCUS ON NOX ENZYMES

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Non permettete mai alla realtà di impedirvi di sognare: voi siete la guida dei vostri sogni e sarete voi a consentire ad essi di realizzarsi...
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INTRODUCTION

The imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant defence, determines a situation called “oxidative stress”. ROS react and oxidize cellular components, such as proteins or DNA, leading to cell death and severe tissue damage. The central nervous system (CNS) is particularly sensitive to oxidative stress because of high oxygen consumption, low antioxidant defense and abundance of lipids, which are prone to oxidation. For this reason a variety of CNS diseases have an oxidative stress component. In the CNS, NOX NADPH oxidases are increasingly recognized as major sources of ROS. Among the seven existing isoforms, the presence of NOX1, NOX2, NOX3 and NOX4 transcripts has been detected in total brain samples. In addition, several immunohistological and in vitro studies have investigated the expression of NOX isoforms in specific CNS regions and cells. From these studies, it appears that NOX1, NOX2 and NOX4 are present in microglia, neurons and astrocytes. Although the physiological role of constitutive NOX expression in the brain is still poorly understood, several researches suggest that they are involved in brain development and function, but most studies indicate that an excessive production of ROS by NOX enzymes participates to the progression of various neurological and psychiatric diseases. The aim of this research project was to investigate the role of NOX2 in the pathogenesis of schizophrenia.
FIRST PART

SCHIZOPHRENIA: CAUSES AND NEUROBIOLOGY
1. INTRODUCTION

A hundred years ago, Emil Kraepelin described the syndrome now called schizophrenia as "dementia praecox". Kraepelin's conceptualization stressed the aspects of severity and chronicity. The syndrome that he defined, "dementia praecox", tended to begin relatively early in life ("praecox") and to produce a pervasive and persistent impairment in many different aspects of cognitive and behavioral function ("dementia"). While Kraepelin repeatedly stressed the diversity of signs and symptoms occurring in dementia praecox, and suggested that abnormalities in volition and affect were especially important, he found a chronic course and a poor outcome to be the characteristic defining features. His concept evolved over time, however, as he received feedback from other experts and his own ongoing clinical experience. Subsequently the subject has continued to fascinate and exasperate researchers in equal measure, generating more heat than light and being notable for memorable quotes rather than durable data. Kraepelin's original formulation was rapidly complemented by the work of Bleuler, who suggested that the term "dementia praecox" should be superseded by "the group of schizophrenias". Bleuler emphasized a different aspect of this large syndrome. While Kraepelin thought about course and outcome, Bleuler pondered the nature of the characteristic symptoms. For Bleuler, the most important symptom was a fragmentation in the formulation and expression of thought, which he interpreted in the light of the associational psychology prevailing at the time and referred to as "loosening of associations." He renamed the disorder "schizophrenia" to emphasize the fragmenting of associations as the fundamental feature of this disorder. The views of a third European psychiatrist, Kurt Schneider, have also been very influential in conceptualizations of schizophrenia. Like Bleuler, Schneider was interested in identifying the main symptoms of schizophrenia. He developed a description of a set of "First Rank Symptoms" (FRS), which he believed to be specific to schizophrenia and diagnostic of it. Schneiderian First Rank Symptoms are specific types of delusions and hallucinations, such as thought insertion, thought broadcasting, delusions of control, or voices commenting. They tend to be tied together by the common thread that the patients perceive themselves as losing control of their thoughts, feelings, and bodies. Schneider's emphasis on these symptoms was
derived from clinical observations and his beliefs about the origin of the disease. These three ways of thinking about schizophrenia - the Kraepelinian, the Bleulerian, and the Schneiderian - coexist in contemporary thinking about the nature of schizophrenia. Individual clinicians tend to vary in the value and emphasis that they place on these three perspectives, sometimes leading to clinical debates as to whether a patient "really has schizophrenia." Some clinicians base their diagnosis primarily on a Kraepelinian emphasis on chronicity and poor outcome, while others stress Bleulerian negative symptoms and thought disorder, and yet others insist on the presence of florid and prominent psychotic symptoms. These differing perspectives reflect a very real debate about the basic essence of schizophrenia. This debate is not likely to reach closure until the disorder can be defined in terms of its pathophysiology and etiology.

The most infamous, that schizophrenia is the “graveyard of neuropathologists” (1), was a statement which, together with critical reviews of the work up to that time (2), marked the nadir of the field. The significant progress which has been made began with CT (Computed Tomography) findings, followed by MRI (Magnetic Resonance Imaging) and by post-mortem studies using improved methodologies and new techniques. The progress allowed Ron and Harvey (3) to charge that “have forgotten that schizophrenia is a brain disease will go down as one of the great aberrations of twentieth century medicine”. In a similar vein, Weinberger (4) stated „20 years ago, the principal challenge for schizophrenia research was to gather objective scientific evidence that would implicate the brain. That challenge no longer exists.” On the other hand, it is undoubtedly an overstatement to claim that there is “an avalanche of consistent evidence of microscopic pathology” (5); the current challenge is to establish the characteristics of the pathological changes (6,7). Clinically, schizophrenia is a devastating mental illness that impairs mental and social functioning and often leads to the development of comorbid diseases. These changes disrupt the lives of patients as well as their families and friends. The onset of schizophrenia can be abrupt or insidious. Most patients undergo a prodromal phase marked by a slow and gradual development of symptoms, such as social withdrawal, loss of interest in school or work, deterioration in hygiene and grooming, unusual behavior, or outbursts of anger. Family members can find this behavior disturbing and difficult to interpret. They may assume that the person is just “going through a phase.”
Eventually, the appearance of active-phase symptoms (e.g., psychosis) marks the disturbance as schizophrenia (8).
2. DIAGNOSTIC FEATURES OF SCHIZOPHRENIA

Schizophrenia is defined as a multidimensional disorder involving emotional and cognitive dysfunctions, which include flat or inappropriate affects, perception and thinking distortions, language and communication, impairments and disorganized or catatonic behaviors. This disorder is characterized by positive and negative symptoms. The positive symptoms include distortions or exaggerations of inferential thinking (delusions), perception (hallucinations), language and communication (disorganized speech), and behavioral monitoring (grossly disorganized or catatonic behavior). These positive symptoms may comprise two distinct dimensions, which may in turn be related to different underlying neural mechanisms and clinical correlations: the «psychotic dimension» includes delusions and hallucinations, whereas the «disorganization dimension» includes disorganized speech and behavior. Negative symptoms include restrictions in the range and intensity of emotional expression (affective flattening), in the fluency and productivity of thought and speech (alogia), in the initiation of goal-directed behaviour (avolition) and in the loss of a sense of pleasure and social withdrawal. These symptoms also must be associated with marked social and occupational dysfunction. Positive and negative symptoms vary in intensity over time; patients may have predominantly one type at any particular time. Cognitive dysfunction, including a decreased ability to focus attention and deficiencies in short term verbal and nonverbal memory, is also a core feature of the illness, which predicts vocational and social disabilities for patients (9). Schizophrenia is also characterized by disorganized thought, which is manifested in speech and behavior. Disorganized speech may range from loose associations and moving quickly through multiple topics to speech that is so muddled that it resembles schizophasia (commonly referred to as “word salad”). Schizophrenia is speech that is confused and repetitive, and that uses words that have no apparent meaning or relationship to one another. Disorganized behaviour may lead to difficulties in performing daily living activities, such as preparing a meal or maintaining hygiene. It also can manifest as childlike silliness or outbursts of unpredictable agitation (8). No single sign or symptom is pathognomonic of schizophrenia; the diagnosis involves the recognition of a constellation of signs and symptoms
associated with impaired occupational and social functioning (10). The criteria of the Diagnostic and Statistical Manual of Mental Disorders (8), used for most research studies, require symptoms to have been present for at least 6 months; there must also be impaired personal functioning, and the symptoms must not be secondary to another disorder (e.g. depression, substance abuse). The peak age of onset is in the third decade, occurring a few years earlier in males than in females (11). The course and outcome are remarkably variable, but better than sometimes believed; only a minority of patients have a chronic, deteriorating course, though many others have enduring symptoms or functional deficits (12). There are five types of schizophrenia: paranoid, disorganized, catatonic, undifferentiated, and residual. Paranoid type is characterized by a preoccupation with one or more delusions or frequent auditory hallucinations; cognitive function and affect remain relatively well preserved. Disorganized type is characterized by confusion, attention and perception problems, disorganized speech and behavior, extreme social withdrawal, as well as flat or inappropriate affect (8). Catatonic type has at least two of the following features: immobility (as evidenced by stupor or catalepsy); excessive, purposeless motor activity; extreme negativism (e.g., resistance to all instructions, maintenance of rigid posture, mutism); or peculiarities of voluntary movement (e.g., posturing, prominent mannerisms, grimacing) or echolalia and echopraxia. A patient is said to have undifferentiated schizophrenia if none of the criteria for paranoid, disorganized, or catatonic types are met (8). Residual type is characterized by the continued presence of negative symptoms (e.g., flat affects, poverty of speech, social withdrawal, bizarre thinking, inactivity, flat affect) and at least two attenuated positive symptoms (e.g., eccentric behavior, mildly disorganized speech, odd beliefs). A patient is diagnosed with residual type if he or she has no significant positive psychotic features. Of note, this classic typing of schizophrenia can be limiting because patients often are difficult to classify. For that reason, an alternative three-factor dimensional model is given. The three factors are psychotic, disorganized, and negative (deficit). The symptoms are categorized as absent, mild, moderate, or severe (8). There is a significant excess of mortality from suicide and natural causes (13). Criminal behavior per se is not a concomitant of schizophrenia, but patients may commit violent acts in response to hallucinations or delusions or because of frustration in social
interactions (14). The lifetime prevalence of suicide is about 10 percent among patients with schizophrenia (15). The lifetime risk of schizophrenia is just under 1% (16). It has a predominantly genetic aetiolog, but no chromosomal loci or genes have been unequivocally demonstrated (17). Schizophrenia has a prevalence of 1% in all cultures and is equally common in men and women (8). Men typically present with the disease in their late teenage years or early 20s, whereas women generally present in their late 20s or early 30s. A family history of schizophrenia is the most significant risk factor. The diversity of neurobiologic findings in schizophrenia is mirrored by the multiplicity of genetic findings. Genetic epidemiologic findings, such as greater concordance with respect to schizophrenia among monozygotic twins than among dizygotic twins and a high incidence of illness among adopted children whose biologic mothers have schizophrenia, point to a significant heritable component that accounts for about 70% of the risk (18). However, schizophrenia does not appear to be monogenic, and there are a number of chromosomal loci for which linkage to the illness has been replicated (19). Single-nucleotide polymorphisms associated with schizophrenia, some of which have been shown to diminish neuronal functions, have been found in genes within these loci, including a G-protein regulator on chromosome 1, a protein on chromosome 6 associated with synaptic structure, a growth factor on chromosome 8 associated with synaptic growth, a response modulator on chromosome 13 that influences N-methyl-D-aspartate (NMDA) glutamate, a receptor on chromosome 15 for acetylcholine (ACh), and an enzyme on chromosome 22 that affects dopamine (DA) metabolism (20,21,22,23,24,25). The glutamatergic, cholinergic, and dopaminergic neuronal mechanisms affected by these genetic factors have been related to various aspects of cognitive dysfunction involving the inability to perceive and remember information (20,25). In addition to the genetic factors, the environmental component of the pathogenesis of schizophrenia, accounting for the remaining 30% of the risk, includes perinatal and childhood brain injury and psychosocial stress over life events such as separation from the family (26,27). Other hypothetical risk factors include season and location of birth, socioeconomic status, and maternal infections. However, data supporting these ideas are inconclusive (28,29). Schizophrenia appears to be a polygenic disorder with environmental and developmental factors mediating a person’s likelihood of
becoming schizophrenic (30). A meta-analysis has shown that patients with schizophrenia are more likely to have experienced obstetric complications, in particular premature birth, low birth weight, and perinatal hypoxia (31). These early environmental hazards appear to have a subtle effect on brain development. In adulthood different environmental stressors act - including social isolation, migrant status, and urban life (32) - and this remains the case even when life events attributable to the incipient psychosis itself are excluded. The way parents raise their children does not seem to have a major impact on future vulnerability, but families do have an important part to play in the course of the illness; patients with supportive parents do much better than those with critical or hostile ones. Collectively, these risk factors point to an interaction between biological, psychological, and social risk factors that drive increasingly deviant development and finally frank psychosis (33,34).
3. NEUROPATHOLOGICAL FINDINGS IN SCHIZOPHRENIA

By 1980, the growing evidence for structural brain changes in schizophrenia provided by CT studies had spurred a return to post-mortem investigations. These have focused on three overlapping areas:

1) first, attempts have been made to confirm whether the alterations were replicable in direct measurements of the brain.

Knowledge of the timing of the brain changes is essential for understanding their aetiological significance. Ventricular enlargement and cortical volume reduction are both present in first-episode cases (35,36,37,38), excluding the possibility that they are a consequence of chronic illness or its treatment. Moreover, adolescents and young adults who are at high risk of developing schizophrenia by virtue of their family history show enlarged ventricles (39) and smaller medial temporal lobes (40), suggesting that the brain pathology precedes the onset of symptoms and supporting a neurodevelopmental model of schizophrenia. It is less clear what happens to the structural pathology after symptoms emerge (12). Neither ventricular nor cortical volume reduction, nor the smaller size of the medial temporal lobe (41), correlate with disease duration, suggesting that the alterations are largely static. However, longitudinal studies, which now span 4-8 years, are equivocal. Some support the view that there is no progression (42,43) whilst others find continuing divergence from controls (44,45). This may reflect a subgroup of subjects with a deteriorating course (46) or who receive high doses of antipsychotics (47), but other studies have not shown such correlations. Overall, the question whether brain pathology in schizophrenia is progressive or static, or even fluctuating, remains controversial, and has an uncertain relationship with the clinical heterogeneity of the syndrome.

2) Secondly, research has sought to clarify the frequency and nature of neurodegenerative abnormalities in schizophrenia, especially to ascertain whether gliosis is present and whether Alzheimer’s disease occurs at an increased frequency.
An altered neurodevelopment may account for the morphological alterations in the brains from schizophrenic patients but does not explain that at least some of those alterations show a progressive course (12,48), which suggests an active neurodegenerative process. On the other hand, both hypotheses (neurodevelopment and neurodegeneration) may be related to each other. Reduced neurotrophic factor signaling, for example, could lead to both an altered development of the CNS and neuronal death because neurotrophic factors are involved in CNS development and neuronal survival. So, hypotheses for neurodegeneration and an altered CNS development in schizophrenia are not mutually exclusive and they even could be complementary. The major brain morphological abnormalities observed in schizophrenic patients are the loss of cortical gray matter, the reduced volume of the amygdala, the hippocampus (49), the frontal and temporal lobes (12, 50,48,51) and ventricular enlargement (12,50,48,49). Some of the pathological findings may be observed since the earlier psychotic episodes and thus they are not likely to be due to chronic antipsychotic medication (12,48) and they are more evident in patients who have suffered multiple episodes of the disease (50). It has also been reported a general decrease of brain mass from the onset of the disorder (49,52,53). Some follow-up neuroimaging studies have reported a progressive ventricular enlargement in schizophrenic patients (12,48). This finding may also be observed during physiological ageing (54), but in schizophrenia it occurs earlier than in normal subjects. The interpretation of pathological findings in schizophrenia should be taken with caution since several factors (such as reduced neuronal size or neuropil, changes in glial cell number or volume) may be responsible for the reduced volume observed in some brain regions in schizophrenic patients. Neuronal loss is one of those factors. Reduced number of neurons has been reported in the anterior cingulate cortex (55), nucleus accumbens (12), hippocampus (12,55) and thalamus (56,57) from people who died with schizophrenia. Other studies have failed to replicate those results (12,55) but it should be considered that total neuron numbers could mask significant decreases in the number of specific cell types within a brain nucleus. In fact, reduced number in specific cell groups such as striatal cholinergic interneurons (58), cortical parvalbumin- containing and calbindin-containing γ-aminobutyric acid (GABA)-ergic cells (59), non-pyramidal neurons in hippocampal sector CA2 (94), cortical NADPH-
diaphorase positive neurons (60,61) and hypothalamic nitric oxide synthase-containing neurons (62), has also been reported in schizophrenia. The medication status of the patients included in those studies is a confounding factor although reduced number of hippocampal neurons is not likely to be due to neuroleptic exposure at the time of death (63), and could be associated to the reduced brain regional volume in first-episode schizophrenics (12,48) and thus neuronal loss in schizophrenia may be related to disease rather than to medication. If neurodegeneration is involved in the pathophysiology of schizophrenia, biochemical or histopathological changes indicative of cell death are expected to be present in schizophrenic brains. As gliosis is a sign of past inflammation, this finding supported a number of aetiopathogenic scenarios for schizophrenia involving infective, ischaemic, autoimmune or neurodegenerative processes. Gliosis has been reported in schizophrenia for at least 20 years (12). Glial proliferation has been found in the subiculum and orbitofrontal cortex of the brains from people with schizophrenia and dementia compared to those from people with schizophrenia without dementia, independently of neuroleptic exposure, although none of those subgroups were significantly different from control samples (64), suggesting that a neurodegenerative process leading to gliosis could be present in a subset of schizophrenic patients (those with severe cognitive impairment). It is also possible that astrocytosis is an epiphenomenon to schizophrenia but it remains to be determined if the co-morbidity of dementia is responsible for neuronal death (leading to gliosis) in this disorder or if the pathophysiology of schizophrenia could in some patients lead to neurodegeneration manifested as dementia. However, most studies have failed to find gliosis in schizophrenic brains (65-67) at least partially due to methodological differences between them. These findings together suggest strongly that gliosis is not a feature of the disease but is a sign of coincidental or superimposed pathological changes. Additionally, gliosis is not always demonstrable or permanent after (postnatal) neural injury, nor does it accompany apoptosis, another process which hypothetically might be involved in schizophrenia (12). Apoptotic mechanisms have also been suggested to occur in schizophrenia (68). In this regard, significantly reduced content of the antiapoptotic protein Bcl-2 (69) and increased Bax/Bcl-2 ratio (associated to susceptibility for apoptotic death) in Brodmann’s Area (BA) 21 (70) and
ultrastructural changes in oligodendroglial cells suggestive of apoptotic death have been found in the brains from people who died with schizophrenia. Those results are not likely to be due to antipsychotic medication (69,70). In spite of the discrepancies between studies, brain histopathological findings in schizophrenia suggest that neurodegeneration may occur in a subset of patients with this disorder (71).

3) The third, and largest, area of research has been to investigate is the cytoarchitecture of the cerebral cortex. Contemporary neuropathological investigations of schizophrenia have been by and large well designed and appropriately analysed. Their renaissance has coincided with the advent of molecular techniques and computerized image analysis, allowing more powerful and quantitative experimental approaches. Nevertheless, it is worth mentioning three limitations which continue to apply, to varying degrees, to most studies. First, few have been carried out according to stereological principles and hence are subject to errors and biases which may be particularly important in this instance, given the subtlety of the alterations being sought. Secondly, research groups have tended to use differing methods, measuring different parameters, and have studied different regions of the brain. It is therefore difficult to know whether inconsistent results reflect genuine pathological or anatomical heterogeneity or methodological factors, or are simply contradictory. Thirdly, sample sizes have continued to be small, leading inevitably to both false-positive and false negative results and meaning that potential complexities, such as diagnosis x gender interactions and discrete clinicopathological correlations, have barely been addressed (12). Since neurodegenerative abnormalities are uncommon in schizophrenia, the question is raised as to what the pathology of the disorder is, and how the macroscopic findings are explained at the microscopic level. This brings to the heart of recent schizophrenia neuropathology research, which has been the increasingly sophisticated measurement of the cortical cytoarchitecture. The focus has been mainly on the extended limbic system (Hipp, dorsolateral prefrontal cortex (DLPFC) and cingulate gyrus), encouraged by suggestions that psychotic symptoms originate in these regions (72,73). Finally, reports of structural abnormalities in the cerebellum in schizophrenia (74) merit further
investigation, given accumulating evidence for its pathophysiological involvement in the disorder (75).

*Cytoarchitectural abnormalities in entorhinal cortex*

An influential paper reported the presence of various abnormalities in the cytoarchitecture and lamination of the entorhinal cortex (anterior parahippocampal gyrus) in schizophrenia (76). The changes were prominent in lamina II, with a loss of the normal clustering of the constituent pre-□ cells, which appeared shrunken, misshapen and heterotopic. Despite extensions and partial replications, the findings remain questionable for several reasons (for example, no normal control group was used; the comparisons were made with brains from 10 patients with other psychiatric or neurological disorders), which are elaborated because of the importance attributed to them in the neurodevelopmental model of schizophrenia. The most serious problem is that no studies have fully allowed for the heterogeneous cytoarchitecture of the entorhinal cortex (77,78) and its variation between individuals (79,80).

*Disarray of hippocampal pyramidal neurons*

A second parameter of cytoarchitectural disturbance in schizophrenia, a disarray of hippocampal pyramidal neurons, has also been given prominence disproportionate to the strength of the data. Normally, pyramidal neurons in Ammon’s horn are aligned, as in a palisade, with the apical dendrite orientated towards the stratum radiatum. Kovelman and Scheibel (81) reported that this orientation was more variable and even reversed in schizophrenia, hence the term “neuronal disarray”. The disarray was present at the boundaries of CA1 with CA2 and subiculum. The basic finding of greater variability of hippocampal neuronal orientation was extended in subsequent studies from the same group (82,83) and independently (84,85). However, none of these studies constitutes true replication. Conrad et al. (83) came closest, but located the disarray at the boundaries of CA2 rather than CA1; Altshuler et al. (82) found no differences between cases and controls, merely a correlation between the degree of disarray and the severity of psychosis within the schizophrenic group; the disarray in the small study of Jønsson et al. (84) was in the central part of each CA field,
and Zaidel et al. (85) found no overall difference in orientation but, in a post hoc analysis, found an asymmetrical variability limited to a part of CA3. Furthermore, there are three entirely negative studies (86,87,88). Thus, even a charitable overview of the data would accept that the site and frequency of hippocampal neuronal disarray in schizophrenia remains uncertain, while a sceptical view would be that the phenomenon has not been unequivocally demonstrated. Certainly, as with the entorhinal cortex abnormalities, it seems inappropriate to place too much interpretative weight on such insecure empirical foundations.

**Location of cortical subplate neurons**

The subplate is a key structure in the formation of the cortex and the orderly ingrowth of thalamic axons (89). Some of the subplate neurons persist as interstitial neurons in the subcortical white matter and contribute to cortical and corticothalamic circuits. Stimulated by the entorhinal and hippocampal cytoarchitectural findings suggestive of aberrant neuronal migration, subplate neurons have been studied in schizophrenia, since changes in the density and distribution of these neurons would probably be a correlate of such a disturbance. Using nicotinamide-adenine dinucleotide phosphate-diaphorase (NADP-d) histochemistry as a marker, these neurons were found to be distributed more deeply in the frontal and temporal cortex white matter in schizophrenics than in controls (60,61). A subsequent survey using additional markers and a larger sample confirmed the observation of fewer interstitial neurons in superficial white matter compartments of DLPFC in schizophrenia (90). These data are more convincing than the reports of entorhinal cortex dysplasias and hippocampal neuron disarray, and the studies are noteworthy for being embedded in the known cellular biology of cortical development. Nevertheless, it would be premature to consider maldistribution of surviving subplate neurons, and by inference aberrant neuronal migration, to be an established feature of schizophrenia.

**Hippocampal and cortical neuron density and number**

A loss of hippocampal neurons is another oftstated feature of schizophrenia. In fact, only two studies have found reductions in neuron density (91,84) and one reported a lower number of
pyramidal neurons (92). In contrast, several have found no change in density (81,92,87,88) and one found a localized increase (93). Since none of these studies were stereological, their value is limited by the inherent weaknesses of neuron counts when measured in this way (94) - although not to the extent that they should be discounted (95). Nevertheless, the fact that the single stereological study that has been carried out found no difference in neuronal number or density in any subfield (96) supports the view that there is no overall change in the neuron content of the hippocampus in schizophrenia. The prefrontal cortex has also been examined. A careful stereologically based study found an increased neuronal density in the dorso-lateral prefrontal cortex (DLPFC) (97,98), and a similar trend was seen for the whole frontal lobe by Pakkenberg (99). The higher packing density identified by Selemon and colleagues affected small and medium-sized neurons more than large pyramidal ones. Other neuronal density studies in the PFC have not produced consistent findings. For example, Benes et al. (100,101) identified a variety of lamina-, area- and cell type-specific differences, whilst unaltered neuronal density has been reported in the motor cortex (88) and DLPFC (102). These discrepancies may be due to anatomical heterogeneity or may be the consequence of differences in the stereological purity of the studies. The total number of neurons in the frontal cortex is not altered in schizophrenia (99), which probably reflects the net effect of anatomical variation in the neuronal density changes within the frontal lobe and/or the trend for cortical grey matter to be thinner in schizophrenia, which compensates for the increased packing density of neurons therein (98,103).

Hippocampal and cortical neuronal size

With the advent of user-friendly image analysis it has become relatively straightforward to measure the size of the cell body of neurons, either by tracing around the perikaryal outline or by measuring the smallest circle within which the soma fits. Three studies, each counting large numbers of neurons, have now identified a smaller mean size of hippocampal pyramidal neurons in schizophrenia (101,88,85). Although different individual subfields reached significance in the latter two studies, the same downward trend was present in all CA fields and in the subiculum. The non-replications comprise Christison et al. (86) and Benes et al. (63),
perhaps because measurements were limited to a restricted subset of neurons. Smaller neuronal size has also been reported in DLPFC, especially affecting large lamina IIIc neurons (104). A degree of anatomical specificity to the size reductions is apparent, since this study found no differences in the visual cortex of the same cases, in agreement with the unchanged cell size found in that region as well as in the motor cortex by Arnold et al. (88) and Benes et al. (100).

**Neuronal morphometric changes in other regions**

Outside the cerebral cortex, consistent cytoarchitectural data are limited to the thalamus. Pakkenberg (105) found markedly lower numbers of neurons in the dorsomedial nucleus, which projects mainly to the prefrontal cortex. A similar finding was observed in the anteroventral nucleus, which also has primarily prefrontal connections, the significant deficit affecting parvalbuminimmunoreactive cells, a marker for thalamo-cortical neurons (106). Whether similar changes occur in thalamic nuclei not intimately related to cortical regions implicated in schizophrenia remains to be determined. In summary, a range of differences in neuronal parameters have been reported to occur in schizophrenia. The abnormalities most often taken to be characteristic of the disorder - disarray, displacement and paucity of hippocampal and cortical neurons are in fact features which have not been clearly demonstrated. In contrast, decreased neuron size, especially affecting neurons in the hippocampus and DLPFC, has been shown fairly convincingly; some studies suggest that the size reduction is accompanied by increased neuron density. The other relatively robust cytoarchitectural abnormality in schizophrenia is in the dorsal thalamus, which is smaller and contains fewer neurons.

**Studies of synapses and dendrites**

Synaptic abnormalities represent a potential site for significant pathology in schizophrenia which would be undetectable using standard histological approaches. The term “synaptic pathology” is used to denote abnormalities in axons and dendrites in addition to those affecting the synaptic terminals themselves. Qualitative studies identified a range of
ultrastructural abnormalities of neuronal and synaptic elements in schizophrenia (107,108,109). However, because of the difficulties and limitations of electron microscopy in post-mortem human brain tissue, especially for quantitative analysis, much contemporary research into synaptic pathology in schizophrenia has adopted a complementary approach whereby the expression and abundance of proteins concentrated in presynaptic terminals, such as synaptophysin, are used as proxies for synapses. This approach has been validated in several experimental and disease states (110,111). For example, in Alzheimer’s disease, synaptophysin mRNA and protein levels correlate inversely with the clinical and pathological severity of dementia (112,113). However, although synaptic protein measurements are widely interpreted as reflecting synaptic density, an assumption almost certainly true in neurodegenerative disorders, in principle changes in synaptic protein expression could instead be due to alterations in synaptic size or number of vesicles per terminal, or to a structural abnormality of the presynaptic region.

*Hippocampal formation*

Synaptic protein determinations in the hippocampal formation (hippocampus and parahippocampal gyrus) in schizophrenia have fairly consistently found levels to be reduced, although not all reach statistical significance for reasons other than just inadequate sample size. First, subfields may be differentially affected (114,115), and localized changes may be masked if homogenized tissue is used. Secondly, the synaptic proteins studied change to varying degrees, probably reflecting their concentration in differentially affected synaptic populations. For example, synaptophysin, which is present in all synapses, shows only slight reductions (116,114,115) whereas SNAP-25 (117) and complexin II (118), which are both concentrated in subsets of synapses, show greater decrements. Furthermore, complexin II is primarily expressed by excitatory neurons, unlike complexin I, which is mainly present in inhibitory neurons and is less affected in schizophrenia (118). Thus, these data suggest a particular involvement of excitatory pathways in this region, a conclusion in keeping with neurochemical studies of the glutamatergic system. A final example to dissect out the nature of hippocampal synaptic involvement in schizophrenia is provided by a study of the expression
of the neuronal growth associated protein-43 (GAP-43), a marker of synaptic plasticity (119). A loss of hippocampal GAP-43 mRNA was found, suggesting that hippocampal synapses may be remodelled less actively in schizophrenia (120). Less attention has been paid to postsynaptic elements of the hippocampal circuitry. However, dendritic abnormalities have been reported, with decreased and aberrant expression of the dendritic microtubule-associated protein MAP-2 in some subfields (121,122).

Neocortex

Two studies have found synaptophysin to be reduced in DLPFC in schizophrenia (123,124). The inferred decrease of presynaptic terminals is complemented by a lower density of dendritic spines (to which many of the synapses are apposed) on layer III pyramidal neurons (125). The pattern of synaptophysin alteration is not uniform throughout the cortex, since levels are unchanged in the visual cortex (114,115) and increased in the cingulate gyrus (126). The suggestion that there is a discrete profile of synaptic pathology in the cingulate gyrus is noteworthy given the other cytoarchitectural and ultrastructural findings in that region, such as increased glutamatergic axons (127,128) and axospinous synapses (129), and deficits in inhibitory interneurons (101) which have not been reported elsewhere. However, further direct comparisons are needed before it can be concluded that the cingulate exhibits a different pattern of pathology.

Thalamus

A marked reduction of the synaptic protein Rab3a from the thalamus was found in a large group of schizophrenics compared with controls (130). These data, in concert with the morphometric and imaging findings, highlight the thalamus as meriting active investigation in schizophrenia (131), a somewhat belated return to the one brain region for which the earlier generation of studies had produced potentially meaningful findings (132).
Striatum

In the striatum, electron microscopy rather than immunocytochemical measurements has continued to be used to investigate synaptic pathology in schizophrenia. Altered sizes and proportions of synapses in the caudate nucleus have been found compared with controls. Imaging of subcortical structures in schizophrenia has produced few clear findings. One firm conclusion is that the striatal enlargement reported in some studies is, unlike the other changes, due to antipsychotic medication (133,134). Indeed, in unmedicated and first-episode patients, caudate volumes are probably reduced (135,136). It is difficult to interpret these findings and integrate them with those in other regions because of the methodological differences and the greater concern about confounding effects of antipsychotic medication in basal ganglia. Nevertheless, they broadly support the view that synaptic organization is altered in schizophrenia. Despite the limitations of the neuronal and synaptic data in schizophrenia, there is an encouraging convergence between the two, at least in the hippocampus and DLPFC, from where most data have been obtained. The grey matter contains an unchanged number of neurons, but the pyramidal neurons are smaller and more densely packed. The cortex is thinner, especially in laminae II and III. The reduced neuron size and increased neuron density are both correlates of a reduced neuropil volume, which in turn reflects abnormalities affecting the axonal and dendritic arborizations of some neurons. For example, there may be less extensive, or otherwise aberrant, synaptic connections formed by incoming corticocortical fibres and by axon collaterals of efferent pyramidal neurons. Glia are unaffected. In particular, the fact that presynaptic and dendritic markers are generally decreased in schizophrenia is in keeping with the finding of smaller neuronal cell bodies, since perikaryal size is proportional to the extent of the dendritic (137,138) and axonal (139, 140) tree. It is also consistent with the findings of increased neuron density, in that dendrites, axons and synapses are the major component of the neuropil and, if the latter is reduced, neurons will pack more closely together (141). Moreover, there is a correspondence with the results of proton MRS (magnetic resonance spectroscopy) and MRS-imaging studies of the hippocampus and DLPFC in schizophrenia, which have shown reductions in signal for the neuronal marker
NAA (N-acetyl-aspartate) (142,143), as one would predict if the constituent neurons are on average smaller and have less extensive axonal arborizations. Parenthetically, the lowered NAA signal is seen in unmedicated (144) and first-episode (145) of schizophrenia. These findings imply that the cytoarchitectural abnormalities seen in post mortem studies, which inevitably are limited to chronic schizophrenia, may also be present at this early stage.
4. PATHOGENETIC HYPOTHESIS

Dopamine (DA) hypothesis

The DA hypothesis of schizophrenia proposes that dysfunction in DA neurotransmission is the underlying cause of the symptoms of the disorder. Specifically, hyperactivity of mesolimbic dopaminergic neurons is suggested to produce the positive symptoms of schizophrenia such as psychosis (149,150). A hypodopaminergic state in the frontal-cortical terminal fields of mesocortical DA neurons has also been proposed to be the basis of negative symptoms (151). Mesolimbic dopaminergic hyperactivity in schizophrenia may be maintained by pre- or postsynaptic mechanisms. Evidence for presynaptic hyperactivity includes excess DA release in response to amphetamine (152,153) and increased L-DOPA decarboxylase levels in schizophrenia (153). Further, amphetamine and related substances such as 3,4-methylenedioxymethamphetamine (MDMA) have been shown to produce psychotic symptoms in healthy subjects. In addition, many patients with schizophrenia experience an exacerbation of psychotic symptoms in response to psychostimulants such as amphetamine and methylphenidate at doses that are not psychotogenic to normal controls. Postsynaptically, an increased number of DA receptors or associated signal transduction elements could also result in heightened sensitivity to DA. Although initially classified into D1 and D2 receptors based on differing biochemical and pharmacological profiles, these DA receptors are now recognized as 2 distinct receptor families. All typical antipsychotics are D2 receptor antagonists, and there is a strong correlation between clinical efficacy (i.e., antipsychotic effect) and the degree of D2 receptor antagonism. Similarly, D2 receptor density, as measured in post-mortem tissue and more recently in in vivo brain imaging studies, has been reported to be increased in schizophrenia (153). However, the effects of long-term antipsychotic treatment on D2 receptors is a common confound in many of the earlier studies. Changes in other DA receptors have also been reported in schizophrenia (154) but many of these studies suffer from similar limitations. However, despite the longevity of this hypothesis there is still no consensus as to the nature of the supposed abnormality or any evidence that dopamine has a
causal role in the disorder (155). There are two main difficulties. First, antipsychotics have marked effects on the dopamine system, seriously confounding all studies of medicated subjects. Secondly, the molecular characterization of the dopamine receptor family has greatly increased the number of potential sites of dysfunction and the mechanisms by which it might occur in schizophrenia. There is no doubt that D₂ receptor densities are increased in schizophrenia, but considerable doubt as to what proportion is not attributable to antipsychotic treatment (156). The dopamine theory of schizophrenia has several flaws, however. First, blockade of dopaminergic neurotransmission does not fully alleviate symptoms of schizophrenia. Second, although positive symptoms of schizophrenia are diminished when dopaminergic neurotransmission is decreased by antipsychotic medications, levels of dopamine metabolites and receptors, when measured in patients before and after treatment, are still generally within the wide range of normal values (157,158). Third, the role of dopamine in the brain is more complex than that of acting as a simple switch for psychotic symptoms. During acute psychotic episodes, many persons with schizophrenia appear to have increased occupancy of receptors in the basal ganglia by DA. However, decreased dopaminergic activity in the cerebral cortex of the frontal lobe may also be one of the factors contributing to the cognitive impairment commonly found in persons with schizophrenia (159). Investigation of the pathophysiology of schizophrenia has therefore extended beyond DA, and researchers exploring the pharmacologic treatment of schizophrenia, while not abandoning DA as a target, have extended their field of inquiry to include other neurotransmitters.

**Glutamate hypothesis**

Ketamine, phencyclidine (PCP) and other NMDA receptor antagonists induce schizophrenic-like symptoms in healthy subjects and precipitate psychoses in patients with schizophrenia who have stabilized (160,161). This has led to the suggestion that schizophrenia may involve hypofunction of NMDA receptors (162,163). Long-term potentiation is disrupted by NMDA antagonists and Kornhuber and colleagues (164) reported increased binding to NMDA receptors in post-mortem frontal cortex of patients with schizophrenia. Similarly, a decreased
release of glutamate has been reported in the frontal and temporal cortices of patients with schizophrenia (165), as have higher blood concentrations of glycine, glutamate and serine (166). Reduced expression of non-NMDA glutamate receptor subtypes in the medial temporal lobe of patients has also been reported (167). Glutamate may also be involved in schizophrenia through its interactions with DA (168), subtle forms of excitotoxicity (163) or the developmental abnormality of corticocortical connections (51). Repeated exposure to PCP has been reported to reduce both basal and evoked DA utilization in the monkey PFC, an effect which persisted even after PCP treatment was stopped (169). Taken together, these findings implicate altered glutamate neurotransmission and NMDA receptor function, in particular, in the negative and cognitive deficits observed in schizophrenia.

**Serotonergic hypothesis**

The serotonergic (5-HT) system has also been frequently implicated in schizophrenia (170). The 2 major classes of psychedelic hallucinogenic drugs, the indoleamines (e.g., lysergic acid diethylamide, LSD) and phenethylamines (e.g., mescaline), are believed to mediate their effects through 5-HT$_{2A}$ receptors (171). Polymorphisms of the 5-HT$_{2A}$ receptor gene are reported to be a minor risk factor for schizophrenia (172). A loss of PFC 5-HT$_{2A}$ receptors along with an accompanying increase in 5-HT$_{1A}$ receptors and a blunted neuroendocrine response to 5-HT$_{2A}$ agonists have been reported in schizophrenia (173). However, recent positron emission tomography studies have been somewhat equivocal in regard to 5-HT$_{2A}$ receptor changes in schizophrenia. Nevertheless, the relatively high affinity of atypical antipsychotics such as clozapine for the 5-HT$_{2A}$ receptor supports a role of 5-HT systems in schizophrenia. As in the case with dopaminergic and glutamatergic animal models, LSD has been shown to disrupt startle habituation and PPI in humans and rats. Further, this effect is believed to be mediated through direct stimulation of 5-HT$_{2A}$ receptors (174). Indeed, the disruptive effects of PCP on PPI have also been proposed to be mediated through indirect activation of 5-HT$_{2A}$ receptors. Interestingly, both LSD and mescaline have been shown to enhance glutamatergic transmission in rats. 5-HT$_{3}$ receptor antagonists have also been shown to attenuate the behavioural hyperactivity caused by PCP, as well as amphetamine administration, but 5-HT$_{3}$
receptor binding sites are not altered in schizophrenia, and the efficacy of 5-HT₃ antagonists in clinical trials of schizophrenia has been variable (174). Despite evidence for altered serotonergic markers in schizophrenia, there is comparatively little evidence of a primary dysfunction of serotonergic systems in this disorder. Moreover, the relevance of LSD administration in animal models is unclear; repeated administration of LSD in humans or animals leads to behavioural tolerance, unlike the situation in schizophrenia. Although many similarities were noted between the effects of LSD-like drugs in humans and the symptoms of schizophrenia, two major differences prompted the widely accepted, but not necessarily justified, conclusion that this class of drugs does not provide a useful model of schizophrenia. First, tolerance was found to develop rapidly to the subjective effects of LSD-like drugs, whereas the symptoms of schizophrenia persist for a lifetime. Second, the hallucinations produced by LSD and related drugs occur typically in the visual modality, whereas hallucinations characteristic of schizophrenia occur in the auditory modality (175).

**GABA hypothesis**

Alterations in GABA neurotransmission in the PFC of patients have also been proposed, on the basis of both theory and experimental evidence (176,177). An interaction between dopaminergic and GABAergic systems in schizophrenia is supported by the fact that GABA neurons in the middle layers of PFC receive direct synaptic input from DA terminals, exert inhibitory control over excitatory output of layer III pyramidal neurons and undergo substantial developmental changes in late adolescence, the typical age of onset for schizophrenia (178). Evidence for reduced GABA uptake sites in the temporal lobe (179), increased GABAₐ receptor binding in superficial layers of cingulate cortex (180) and reduced gene expression for glutamic acid decarboxylase in the PFC (181) provides direct support for GABAergic involvement in this disorder. In animal studies, the GABAₐ receptor antagonist picrotoxin has been shown to reduce PPI in rats when injected into the medial PFC (176). Further, pre-treatment with the DA antagonist haloperidol antagonized this effect, suggesting that blockade of GABA receptors in PFC impairs sensorimotor gating in a DA-dependent manner. However, because of the lack of any other reported GABA-induced behavioural
deficits related to schizophrenic symptoms, further studies are required to establish the relevance of GABA-based pharmacological models of schizophrenia.
5. PHARMACOLOGICAL TREATMENT

An acute psychotic episode in a person with schizophrenia appears to reflect a convergence of pathologic processes that can include an increase in the neurotransmission of dopamine (perhaps in response to stress), one or more genetic factors that alter the neurotransmitter mechanisms regulating the activity of cortical neurons, and nongenetic factors that have caused a loss of neurons and their connections. The result is a brain that is hypersensitive to stimuli and unable to regulate its response through normal inhibitory mechanisms. The decrease in the number of neurons and interneuronal connections that store and process information further diminishes the ability of the brain to sort the incoming information into what is known and what is unknown. Persons with schizophrenia therefore experience the world as overwhelming and commonly form the delusion that an evil force is controlling them or the world around them, or both. The pharmacologic approach to this manifestation of psychosis has centered on the neurotransmitters that control the response of neurons to stimuli. Neurons that store and process information, such as the pyramidal neurons found in the cerebral cortex, are regulated by many other neurons. Inhibitory interneurons, which regulate cortical neurons, are a primary source of such regulation. The interneurons monitor and inhibit pyramidal-neuron activity. The activity of both pyramidal and inhibitory neurons is further modulated by dopaminergic neurons, as well as by serotonergic, cholinergic, and noradrenergic neurons, which send afferents into the cortex (Fig. 3). The receptors for dopamine, serotonin, and acetylcholine provide additional targets for newer antipsychotic drugs (146). Effective pharmacologic treatment of schizophrenia has been available since the 1950s. In the early 1950s, the term “neuroleptic” was introduced to denote the effects of chlorpromazine and reserpine on laboratory animals. It was intended to distinguish their effects from those of sedatives and other CNS depressants (147). Although “neuroleptic” is still used synonymously with “antipsychotic,” the term now usually refers to first-generation antipsychotics that confer an increased risk of extrapyramidal side effects, such as dystonic reactions (e.g., fixed upper gaze, neck twisting, facial muscle spasms), parkinsonian symptoms (e.g., rigidity, bradykinesia, shuffling gait, tremor), and akathisia (e.g., inability to sit still,
restlessness, tapping of feet). Tardive dyskinesia, which is a chronic disorder of the nervous system characterized by involuntary jerking movements (primarily of the face, tongue, and jaw), often is considered an extrapyramidal side effect. However, it is actually a separate and mechanistically different phenomenon. The term “atypical antipsychotic” refers to newer antipsychotics that confer less risk of extrapyramidal side effects than traditional antipsychotics. Nonadherence to medications is a significant problem; in a recent study, 74% of patients discontinued their medication within 18 months (148). Nonadherence often leads to relapse of symptoms. Atypical antipsychotics were initially thought to help with adherence because of their lower rate of neurologic side effects.

FIRST-GENERATION ANTIPSYCHOTIC AGENTS: the first antipsychotic, or neuroleptic, drug used to treat schizophrenia was chlorpromazine. Its antipsychotic effects were identified incidentally, when it was tested as an antihistamine in subjects who happened to have schizophrenia. Later research established that blockade of dopamine receptors is the therapeutic mechanism for the effectiveness of chlorpromazine in schizophrenia and prompted the development of increasingly potent dopamine antagonists. Drugs such as haloperidol are more than 100 times as potent as chlorpromazine as antipsychotic agents, but they also are more likely to have parkinsonian side effects caused by dopamine blockade in the basal ganglia. Initial administration of first-generation antipsychotic drugs such as haloperidol and chlorpromazine in a patient with schizophrenia results in an immediate blockade of dopamine D2 receptors and a partial antipsychotic effect. Further therapeutic effects develop over the course of six to eight weeks, a period of time that correlates with decreased release of dopamine from presynaptic terminals. About 20% of patients have complete remission of their symptoms. Most patients have some response but also have continuing symptoms. The most obvious side effects are involuntary movement disorders arising from the extrapyramidal system, many of which mimic the effects of Parkinson’s disease and reflect the blockade of dopaminergic transmission between the dopaminergic neurons of the substantia nigra and the dorsal neostriatum. Symptoms include dystonia, akathisia, bradykinesia, and tremor. Elderly patients may be at increased risk for hip fractures as a result of drug associated movement
disorders. As with all patients who have involuntary movement disorders, patients with schizophrenia are distressed but generally have difficulty describing the problem. Hence, they may appear in physicians’ offices with vague reports of their symptoms. Akathisia, a severe state of restlessness that is difficult to distinguish from agitation, is a major cause of non-compliance with the drug regimen. Bradykinesia, decreased spontaneous movement and slowed voluntary movement, mimics the effects of depression. Tardive dyskinesia, a choreoathetotic movement disorder, develops in about 30 percent of patients, generally after several years of treatment. Orofacial movements such as grimacing are common manifestations. Tardive dyskinesia does not respond to anticholinergic agents; it resolves slowly after the withdrawal of first-generation drugs, but it may be irreversible. Death caused by the administration of antipsychotic drugs is rare but can occur through several mechanisms. Temperature dysregulation can lead to a severe neuroleptic malignant syndrome, in which the patient’s temperature exceeds 40°C (104°F) and brain death ensues. The occurrence of such an extreme outcome is related to both environmental. A prolonged QT interval is a side effect of several antipsychotic drugs, and the possibility of this abnormality limits the dose of thioridazine, in particular. To what extent a prolonged QT interval predisposes a patient to the potentially fatal torsade de pointes arrhythmia is unknown, but the incidence of sudden death among patients treated with antipsychotic drugs is 0.015% per year – about twice the rate reported in the normal healthy population.

SECOND-GENERATION ANTIPSYCHOTIC AGENTS: a second generation of antipsychotic agents has been introduced into clinical practice over the past 15 years in an attempt to improve therapeutic effects and decrease the side effects associated with first-generation dopamine-blocking drugs. All second-generation drugs share the D2 dopamine-receptor antagonism of first-generation drugs, but second-generation drugs are less tightly bound to the D2 receptor, and D2-receptor antagonism is no longer the sole therapeutic mechanism. Hence, there are similarities in the general scope and time course of the effects of first- and second-generation drugs, but there are clinically important differences in both the therapeutic effects and the side effects. Clozapine is the first atypical antipsychotic drug, so designated
because it has antipsychotic effects without the adverse effects on movement of the first-generation drugs. In addition, clozapine has enhanced therapeutic efficacy, as compared with the first-generation drugs. Therefore, it was introduced into clinical practice despite a serious known adverse effect: an increased incidence of agranulocytosis. Patients taking clozapine must undergo frequent monitoring of the leukocyte count (weekly for the first six months and every two weeks thereafter, including the first four weeks after the patient has discontinued the drug). Nevertheless, for 30% of patients who do not have a response to other treatments, clozapine has substantially enhanced therapeutic effects that justify its use. Clozapine reduces suicidal behavior, although a decrease in the rate of death by suicide has not yet been fully established. Clozapine has significant antagonist effects at D1, D2, and D4 dopamine receptors, as well as at norepinephrine and serotonin receptors. An unexpected effect is that patients who are smokers and who have a response to clozapine also decrease their cigarette smoking. It has been hypothesized that heavy smoking among persons with schizophrenia is an attempt at self-medication, and indeed, nicotine does briefly improve several aspects of brain function. Clozapine increases the synaptic release of acetylcholine, a fact that may account in part for its enhanced therapeutic effect, and concomitantly provides patients with an alternative to the use of nicotine, which is a cholinergic agonist.

Newer Second-Generation Agents: the enhanced antipsychotic action of clozapine was initially thought to be due to its antagonism of both D2 dopaminergic and 5-hydroxytryptamine (5-HT) of type 2A (5-HT2A) serotonergic receptors. Drugs with a similar combined antagonism - including risperidone, olanzapine, quetiapine, and ziprasidone - are all effective antipsychotic agents. Like clozapine, these drugs have an efficacy that is equivalent to or exceeds the efficacy of first-generation antipsychotic agents, without many of the extrapyramidal effects of the first-generation drugs. These newer agents also entail a greatly reduced risk of tardive dyskinesia. Their increased efficacy with respect to negative schizophrenic symptoms is particularly noteworthy, and the rate of relapse is significantly less than that with the first-generation drugs. Other second-generation drugs have a different putative mechanism, involving refinements of action at dopamine receptors. Aripiprazole was characterized by
mixed agonism and antagonism at dopamine receptors in preclinical studies, and investigators proposed that the drug enhanced low levels of dopaminergic transmission, thus improving cognition, but blocked higher levels of transmission that might cause psychosis. Aripiprazole also has effects at serotonergic receptors. Amisulpride is an antagonist at D2 and D3 dopamine receptors. Both aripiprazole and amisulpride have antipsychotic effects that are associated with a lower risk of movement disorder than that associated with first-generation agents. Although only clozapine causes agranulocytosis in a substantial proportion of patients, many second-generation drugs produce clinically significant weight gain. Diabetes mellitus has been increasingly reported in patients treated for more than five years with second-generation antipsychotic agents, presumably in association with weight gain; there is also some evidence of the development of insulin resistance. In a few cases, life-threatening ketoacidosis has occurred. Cholesterol levels increase by 10% after 14 weeks of treatment with olanzapine. Ziprasidone and amisulpride at recommended doses cause less weight gain than do other antipsychotic drugs. Second-generation antipsychotic agents can sometimes induce obsessive–compulsive symptoms, which may reflect antagonism of serotonergic neurotransmission. The extent to which second-generation antipsychotic agents improve cognition in patients with schizophrenia is controversial. First-generation antipsychotic agents have moderate effects on cognition, improving the patient’s ability to pay attention to tasks. Studies comparing first-generation and second-generation drugs show that about 30 to 70% of patients receiving second-generation drugs have improvement on neuropsychological tests of cognitive function, particularly in assessments of attention and short-term memory. Improvement in these cognitive functions is seen in only 30% of patients receiving first-generation drugs. The additional improvement in those receiving second-generation antipsychotic drugs, however, may not translate directly into an improved quality of life for all patients. Furthermore, whether the difference in improvement in cognitive functions reflects the differential effects of the two groups of drugs on dopamine or on other neurotransmitters such as serotonin or acetylcholine is unknown.
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180.
SECOND PART

SCHIZOPHRENIA AND OXIDATIVE STRESS:
THE NOX ENZYMES IN THE CENTRAL
NERVOUS SYSTEM
I) EVIDENCE FROM PATIENT STUDIES AND ANIMAL MODELS

1. Introduction

The involvement of oxidative stress in the pathogenesis of schizophrenia was first proposed in 1954 by Hoffer, who treated his patients using an antioxidant therapy (1). This idea has been then confirmed by subsequent studies, and in recent years, the origin and the impact of reactive oxygen species (ROS) has elicited increased interest in the field of schizophrenia research. In agreement with patient studies, signs of oxidative stress have also been observed in animal models following social isolation or treatment with NMDA receptor antagonists, such as phencyclidine and ketamine.

2. Patient studies

Direct measurement of ROS in patients represents a technical challenge, but signs of oxidative stress can be evaluated by measuring the amount of antioxidant enzymes (SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase) and molecules (albumine, bilirubine, uric acid, vitamine C and E) in plasma and erythrocyes or in post mortem samples. The plasmatic levels of antioxidants are significantly decreased in schizophrenic patients, indicating an increased vulnerability to oxidative stress (2). Similarly, an impaired function of antioxidant enzymes and SOD in particular, is observed at the onset of psychotic symptoms (3). In contrast, it appears that SOD activity augments in more advanced phases of the disease (4). This increase has been proposed to underlie a compensatory mechanism aiming at neutralizing the excessive generation of ROS (5). It has also been suggested that different alterations of antioxidant defence are correlated with the manifestation of the various symptoms of schizophrenia (4). Presence of oxidative stress is similarly characterised by increased levels of lipid peroxidation products detected in plasma, cerebrospinal fluid and post mortem brain sections of schizophrenic patients (2, 6). Although the role of oxidative stress is still unclear, it is thought that oxidative damage occurs very early in the course of the
disease (7-9) leading to oxidation of neuronal membranes and subsequent alteration of neurotransmission and signal transduction, whose schizophrenia symptomatology would be the manifestation (2, 4).

3. Animal models

Several animal models have been developed based on the concept that schizophrenia is a neurodevelopmental disorder. The model of post weaning social isolation consists in the deprivation of the social interactions structuring the normal behaviour of mammals. This is a non-pharmacological and non-invasive method aiming at reproducing the long-term effect of environmental adverse events on brain development during the early life period (10). Indeed, rearing rodents in persistent social isolation after weaning induce long-lasting behavioural, neurochemical and cognitive alterations similar to several symptoms of schizophrenia (11). Behavioural anomalies in isolated animals include locomotor hyperactivity to a novel environment, aggressiveness, impairment in the prepulse inhibition and cognitive deficits (12-14). Neurochemical abnormalities are characterised by alteration of neurotransmitter levels (glutamate, GABA, dopamine) in the prefrontal cortex, nucleus accumbens or hippocampus and contribute to the development of neuroanatomical changes (10). In particular, a reduction in the expression of GABAergic neuron markers parvalbumin and calbindin in specific regions is a feature usually present in isolated rat brains (15,16). In the same manner, signs of oxidation, such as the presence of lipid peroxidation products and nitric peroxide metabolites are elevated in mouse brain after social isolation stress (17, 18), while increased activation of antioxidant enzymes can be detected in isolated rat brain (19).

- Post weaning social isolation

Several animal models have been developed based on the concept that schizophrenia is a neurodevelopmental disorder. The model of post weaning social isolation consists in the deprivation of the social interactions structuring the normal behaviour of mammals. This is a non-pharmacological and non-invasive method aiming at reproducing the long-term effect of environmental adverse events on brain development during the early life period (10). Indeed,
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- NMDA antagonist model

It was discovered in 1959 that subanesthetic doses of the NMDA receptor antagonist phencyclidine provoke psychotic symptoms, mood changes and perceptual abnormalities in normal subjects, similar to those observed in schizophrenic patients (20). Phencyclidine was used as a "dissociative anesthetic" since it causes sedation, analgesia, induction and maintenance of general anesthesia by provoking a sense of detachment from the body and the mind. However, the manifestation of numerous adverse symptoms (such as delirium, excitement, disorientation, and confusion) has made phencyclidine administration unsuitable for medical care (21). Phencyclidine is still used for research purpose on animals to mimick schizophrenic symptoms (22-24). Other molecules structurally related to phencyclidine have been also discovered, such as dizocilpine (MK-801) and ketamine. Adverse effects of ketamine are less important than phencyclidine and for this reason it is still employed for emergency care (25). In addition, controlled administration of ketamine in healthy volunteers has emerged as a useful tool to study schizophrenia pathogenesis in humans. Similarly, ketamine is able to induce many of the behavioural and neurochemical abnormalities of schizophrenia in rodents. For this reason, acute and chronic administration of ketamine is a validated technique for
schizophrenia modelling (26). Although it is not possible to know whether animals experience hallucinations or delusions, a marked alteration of their motor behaviour is usually manifested immediately after NMDA-receptor antagonist injection (27, 28). This effect on locomotion is mainly due to the activation of the prefrontal cortex and its connected limbic structures. Since the activation of these brain regions is also critical for schizophrenic symptoms in humans, changes in locomotion are considered as a read-out for quantifying the severity of behavioural alterations (29, 30). In the same way, increased levels of glutamate and dopamine have been detected in mouse prefrontal cortex following ketamine injections (31). Moreover, chronic exposure to ketamine and phencyclidine is able to modify neuronal connections and plasticity. In particular, cortical fast-spiking inhibitory interneurons present phenotypic changes in brain regions involved in the development of schizophrenia-like symptoms (32, 33). Signs of oxidative stress are also present in animals treated with NMDA-receptor antagonists as seen in patients. More specifically, an elegant study using in vivo microdialysis showed that ketamine injections cause immediate formation of free radicals in the mouse brain (34) and oxidative damage in rats (35). Moreover, treatment with antioxidants attenuated neuronal necrosis induced by MK-801 in the rat brain (36).
4. References


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II. THE NOX ENZYMES IN THE CENTRAL NERVOUS SYSTEM

1. Introduction

Oxidative stress in the central nervous system (CNS) is a major contributor to disease and aging. Until recently, mitochondria were thought to be the principal source of ROS in these contexts (1). However, there is increasing evidence that NADPH oxidase (NOX) enzymes might also play a role in ROS production in the CNS. So far, the involvement of NOX enzymes has been investigated with respect to cerebrovascular diseases (2,3) and inflammatory processes regulated by NOX2 in microglia (4). However, recent data indicate that NOX enzymes are widely expressed in the CNS, possibly fulfilling a large range of physiological functions and being implicated in a variety of CNS diseases. The ROS-generating NOX enzymes are electron transporters. The electron from cytoplasmic NADPH is transferred across biological membranes through NOX enzymes and bound to oxygen in the extracellular space or in the lumen of intracellular organelles, via intermediate flavin adenine dinucleotide (FAD) and heme prosthetic groups. Superoxide (O2-) is generally thought to be the primary product of the electron transfer, but other downstream ROS, in particular hydrogen peroxide (H2O2), are also generated (5). Seven NOX genes have been identified: NOX1-5, DUOX1 and 2. Since little is known about the role of NOX5 and DUOX1-2 in the CNS, in this part, I will focus exclusively on NOX1, NOX2, NOX3 and NOX4. The first NOX enzyme to be discovered was NOX2 in phagocytic cells and, for this reason, it is also known as gp91phox (phox: phagocyte oxidase). The transmembrane NOX2 protein produces ROS upon activation by the interaction with another transmembrane protein, p22phox, as well as the cytosolic subunits, p47phox, p67phox, p40phox, and one of the small Rho GTP-binding proteins, Rac1 or 2. The other NOX isoforms (i.e. NOX1, NOX3 and NOX4) are also transmembrane proteins associated with p22phox, but have a different mechanism of activation. The NOX1 enzyme requires the interaction with its cytosolic subunits, NOXA1 and NOXO1, and with Rac1 or 2. NOX3 needs to interact with NOXO1, but it is not clear whether the binding with NOXA1 and Rac proteins is necessary. In contrast, NOX4 seems to be
constitutively active, without the presence of cytosolic subunits, while its regulation by Rac is not yet clear (5, 6) (Figure 1).

![Diagram of NADPH NOX enzymes](image)

**FIG. 1. Schematic overview of NADPH NOX enzymes.** NOX enzymes are transmembrane electron transporters. NOX isoforms have similar structures, but different activation mechanisms. To produce superoxide ion (O₂), NOX1 requires the interaction with p22phox, the cytosolic subunits, NOXO1 and NOXA1, and the small Rho GTP-binding protein Rac. NOX2 needs to interact with p22phox, phosphorylated p47phox, p67phox, and Rac. NOX3 activation is similar to that of NOX1, although it is not clear whether the interactions with NOXA1 and Rac are necessary. NOX4 interacts only with p22phox and is thought to be constitutively active.

The conditions in which NOX is regulated/activated in the CNS are not elucidated. Indeed, the specificity of NOX regulation in the CNS could come from putative NOX-interacting proteins, which have a preferential expression in the CNS, such as NOXO1γ and Rac3.

**NOXO1 γ:** The NOXO1 γ splice variant is strongly expressed in the fetal brain, while it is not detected in the adult (7). Thus, it is not known whether this splice variant is involved in brain development.

**Rac3:** In addition to the Rac GTPases Rac1 and Rac2, a third homologue, Rac3, is predominantly expressed in neurons (8). Given its high sequence similarity with Rac1, it is possible that Rac3 is involved in neuronal NOX activation. However, to our knowledge, this
question has not been yet addressed experimentally. The subcellular localization of NOX enzymes can also influence the ROS-mediated signaling and cellular functions. However, in most of cases, the question of the subcellular localization of NOX isoforms is still a matter of debate. This uncertainty is, at least in part, due to the lack of high quality antibodies. Most of the data concerns NOX2 in granulocytes, where the enzyme localizes predominantly to intracellular granules in quiescent cells, but is translocated to the plasma membrane and/or the phagosome in response to cell activation (9). Similarly as seen for phagocytes, it appears that in neurons there is a membrane translocation of cytosolic NOX2 subunits upon cell activation (10, 11). In addition, it has been observed that in hippocampal neurons, NOX2 enzyme is present in synaptic sites (11). To our knowledge, no studies have been published about the localization of other NOX isoforms in CNS cells. However, based on observations in vitro on other cell type, it has been suggested that NOX4 might localize to the endoplasmic reticulum (12, 13, 14) or vinculin to focal adhesions (15), while NOX1 might preferentially localize to the plasma membrane (13, 15), possibly to caveolae (15). Very little is known about NOX3 localization.
2. Expression of NOX enzymes in the CNS and in its cells

The family of NOX enzymes is widely distributed in a variety of tissues, but very high expression levels can be found in specific organs or cell types (e.g. NOX1 in the colon, NOX2 in phagocytes, NOX3 in the inner ear, NOX4 in the kidney). It also appears that many cells can express several NOX enzymes with non-redundant functions. This non-redundancy of NOX function is most likely explained by different subcellular localization and activation mechanisms of the different NOX isoforms (5, 16, 17).

Expression of NOX enzymes in the brain and in specific CNS areas

The presence of NOX1, NOX2, NOX3 and NOX4 transcripts has been identified in total brain samples (18, 19, 20). In addition, several studies have investigated the expression of NOX isoforms in specific CNS regions. Most data concern NOX2, however there is evidence on the CNS localization of NOX1, NOX4 and possibly also NOX3. Although available studies do not provide a comprehensive description of the CNS distribution of NOX enzymes, it appears that: i) several NOX isoforms are co-expressed in various CNS regions and ii) at least under some circumstances, NOX expression in certain CNS regions might be inducible, rather than constitutive.

Expression of NOX enzymes in specific cells of the CNS

Evidence of the expression of NOX isoforms in specific cell types of the CNS comes mostly from in vitro studies on derived primary cultures. It appears that NOX1, NOX2 and NOX4 are present in neurons, astrocytes and microglia, while the localization of NOX3 is not known. Unfortunately, the relative amount of different NOX enzymes and their functional activity in different brain cells has not been comparately studied.

Physiological function of NOX enzymes in the CNS and in its cells

Physiological concentrations of ROS are necessary for the proper function of several biological processes. Activation of NOX enzymes is required in different processes such as host defense,
biosynthesis or cellular signaling cascades (21). Genetic and pharmacological studies have allowed the identification of mechanisms regulated by NOX-dependent ROS production. Genetic deletions or mutations in NOX enzymes occur naturally in humans or can be generated in animals and have helped to understand some of the roles of certain isoforms. Most of the chemical inhibitors used currently in pharmacological studies targeting NOX activity are not NOX-specific (for a review (22) and Jaquet et al.). Some of the compounds that have been used in various studies are (Fig.2):

*Diphenyleneiodonium* (DPI) is a potent blocker of electron transporters. In addition to inhibiting NOX enzymes, DPI inhibits xanthine oxidase, nitric oxide synthetase and mitochondrial electron transport.

*Apocynin* is an antioxidant, which under certain circumstances can be metabolized into a NOX inhibitor (22). In most in vitro studies with CNS cells, high concentrations of apocynin (>100 μM) have been used and have displayed antioxidant effects. However, in animal models apocynin appears to be efficacious even at relatively low concentrations (i.e. 2.5-5mg/kg; (23, 24, 25), which argues against a ROS scavenger effect and in favor of an inhibitor effect.

*Atorvastatin*, in addition to its effect on cholesterol synthesis, it inhibits isoprenylation of Rho GTPases and, through this mechanism, decreases the activation of Rac-dependent NOX enzymes.

*AEBSF* is a serine protease inhibitor, which also inhibits NOX enzymes, albeit with a relatively low potency. It is not clear whether AEBSF acts directly or indirectly on NOX enzymes.

*gp91ds-tat* is an inhibitory peptide based on an analogue sequence within gp91phox/NOX2. gp91ds-tat appears efficacious in several animal models; however, its specificity has not been rigorously proven.
Neurological functions of NOX enzymes in the CNS

Most of the information on the physiological functions of NOX enzymes in the CNS has been obtained by using NOX-deficient mice and can be summarized as follows:

NOX1: In mice, genetic deletion of NOX1 leads to decreased blood pressure and protects against aortic dissection in response to angiotensin II (26, 27, 28). With respect to neurological functions, it has been reported that NOX1-deficiency does not alter locomotor activity or movement coordination. Nevertheless, NOX1 enhances the sensitivity to inflammatory pain. Thus, NOX1 deficiency decreases thermal and mechanical hyperalgesia during inflammation, but does not affect nociceptive responses to heat and mechanical stimuli (29). It is not clear whether these effects on pain are due to NOX1 in the central or the peripheral nervous system.

NOX2: The best documented human pathology linked to a loss of function of a NOX enzyme is chronic granulomatous disease (CGD). It is an immunodeficiency caused by mutations in
one of the genes of the NOX2 enzyme complex such as NOX2/gp91phox, p47phox, p67phox, p22phox (30). Since mice that are deficient for the NOX2 enzyme complex display pathological features similar to CGD patients, they have been widely used as experimental models (31, 32). Data about the possible role of NOX2 in CNS functions arise from studies on CGD patients and CGD mice. Among CGD patients an elevated prevalence rate of cognitive deficits is found (33). It has been argued that the chronic disease in CGD patients, in particular recurrent infections, is not the cause of the cognitive deficits (33). This is also in line with the observation that NOX2- and p47phox-deficient mice, which under SPF conditions generally do not display signs of the disease, show impaired memory (34). Thus, it is likely that NOX2 plays a role during CNS development and/or in CNS function. Supporting this hypothesis, it is known that ROS are important signaling molecules involved in mechanisms underlying synaptic plasticity and memory formation (35).

NOX3: Mutation of NOX3 in mice causes vestibular defects due to altered otoconia formation in the inner ear (36). A similar effect has been also observed in NOXO1- (37) and p22phox-mutated mice (31). To our knowledge, analyses of neurological function have not been performed in these mice.

NOX4: No information has been reported on the effects of genetic alteration of NOX4 in human or animals.

**Cellular functions of NOX enzymes in the CNS**

It has been reported that NOX enzymes have physiological and pathological effects on many cell types within the CNS. However, the precise mechanisms of action are not yet fully understood. ROS produced by NOX enzymes can directly influence cellular functions, by inducing the oxidation of proteins and subsequently their structural and functional changes (e.g. hydrogen peroxide-dependent cysteine-modifications in tyrosine phosphatases, transcription factors or ion channels). However, other processes may also occur, including superoxide interaction with nitric oxide (i.e. nitric oxide depletion, peroxynitrite production),
electrogenic effects of NOX activity (i.e. plasma membrane depolarization), or impact on pH homeostasis (21).

**Neurons: differentiation, signaling and death**

*i) Neuronal differentiation*

Reactive oxygen species might be involved in neuronal differentiation during development (38, 39). In this context, NOX enzymes have been implicated in nerve growth factor (NGF)-induced neuronal differentiation of PC12 cells (40, 41, 42) and mesenchymal stem cells (43). During neuronal growth, production of H2O2 by NOX enzymes seems to influence NGF signaling through regulation of tyrosine phosphorylation and activation of the transcription factor AP-1 (42). Consequently, ROS-mediated protein activity and gene expression modulate the development of neuronal cells. In particular, it has been reported that ROS production by NOX enzymes can be involved in different aspects of neurite outgrowth (40, 44). Thus, NOX enzymes can participate in neuronal maturation and differentiation during brain development.

*ii) Neuronal signaling*

**Regulation of membrane potential and H+ fluxes**

Production of ROS by NOX enzymes involves electron transport across biological membranes and, hence, causes cellular depolarization. For each electron transported across the membrane, one H+ ion is left in the cytoplasm. Thus, in order to avoid H+ accumulation and cytosolic acidification, H+ extrusion occurs throughout proton channels. These mechanisms have been studied on NOX2 in phagocytes (45, 46, 47), however, they might be also important in neurons, since neuronal activity is dependent on the plasma membrane potential. Expression of voltage-gated proton channels has been first detected in snail neurons, but is also present in rat hippocampal neurons, where it contributes to regulation of pH homeostasis and action potentials (48). Interestingly, it has been observed that expression of proton channels and NOX2 often occur in parallel (48). However, further analysis needs to be done in order to
better understand the involvement of NOX enzymes in the regulation of membrane potential and H+ fluxes in neurons.

**Angiotensin II receptor**

Angiotensin II is an oligopeptide involved in cardiovascular homeostasis and regulation of blood pressure, mainly through the interaction with the angiotensin II type 1 receptor (AT1R) (49). Release of ROS mediates angiotensin II signaling in the vasculature (50), and NOX enzymes have been implicated in such mechanism (51). In particular, the effect of angiotensin II on NOX enzymes in cerebral vasculature has been investigated (3). It appears that angiotensin II signaling is dependent on NOX activation also in neurons. It has been demonstrated that NOX2-derived ROS are responsible for the entrance of Ca2+ currents in angiotensin II-stimulated neurons (52, 53). As a consequence, NOX2-derived ROS influence AT1R signaling cascades (54, 55, 56, 57), neuronal activity (58), and CNS-regulated cardiovascular responses (59, 60, 61). In addition, it has been observed that NOX enzymes are expressed by neurons of cerebral areas implicated in blood pressure control (i.e. neurons of tractus solitarius (52), lamina terminalis (61) or hypothalamus and brain stem areas (58), where they can influence angiotensin II signaling via ROS production. If in physiological conditions, NOX activity serves as regulator of angiotensin II effects, in pathological conditions, such as an excess salt diet (62, 63) or exposure to hypertensive agents (10), an altered NOX activity and excessive ROS production induced by angiotensin II can contribute to neurogenic hypertension and related cerebrovascular diseases. Interestingly, angiotensin II-induced NOX activation can enhance neuronal death triggered by 6-hydroxidopamine (64, 65).

**NMDA receptor**

The ionotropic NMDA-receptor (NMDA-R) is activated mainly by the neurotransmitter glutamate (66). Whereas in physiological conditions NMDA-receptor activity is involved in mechanisms such as neuronal growth and synaptic plasticity, its excessive excitation in several CNS diseases may cause neuronal death. There are two putative connections between NOX enzymes and the NMDA receptor:
i) NOX enzymes may regulate the NMDA receptor

A tight regulation of NMDA receptor function is required for cell maintenance and survival. In addition to its ligand-dependent activation, the NMDA receptor appears to be modulated by the redox potential: the reduction or oxidation of cysteine residues on its subunits modifies channel conformation and promotes Ca2+ currents entrance or blockade, respectively (67). It has also been suggested that the precise effect of ROS on NMDA receptor activity is dose dependent (68). Relatively little is known about the role of NOX enzymes as being the source of the redox regulation of the NMDA receptor. In one study, the non-specific NOX inhibitor DPI prevented the loss of NMDA receptor-dependent long-term potential induced by β-amyloid, thereby, suggesting that NOX-inhibition enhances NMDA receptor function (69).

ii) the NMDA receptor may regulate NOX enzymes

Activation of the NMDA receptor may lead to NOX2-dependent ROS production in neurons, and thereby activates redox-sensitive signaling cascades including ERK1/2 phosphorylation (70, 71). In apparent contradiction, there is also evidence suggesting that the NMDA receptor antagonist ketamine increases NOX2 activity (18, 19); however, this is most likely an indirect effect mediated via neuronal production of interleukin-6 (23, 72).

iii) Neuronal Death

Most studies on the role of NOX enzymes in neurons have focused on the induction of cell death caused by excessive ROS production. There are many situations where NOX-dependent neuronal death occurs. In the majority of the cases, the role of NOX enzymes has been assessed by using non-specific NOX inhibitors and NOX2 has been indicated as the main source of ROS-dependent cell death. However, compounds used in these studies are not specific inhibitor of NOX activity, and they do not target selectively NOX2. Given that neurons express also other NOX isoforms, their role in neuronal death cannot be excluded.
Microglia

Microglia are resident macrophages of the CNS involved in host defense. Usually present in a ramified resting form, they become amoeboid and active in response to several insults (e.g. damaged neurons, pathogens, altered protein accumulation), and release cytotoxic and inflammatory mediators, such as ROS, nitric oxide and cytokines (73). The production of ROS in activated microglia following many different types of stimuli was associated with NOX2 expression, but recent reports suggest that NOX1 and NOX4 can also play a role. Several physiological processes which are involved in the activation of microglia are regulated by NOX-dependent ROS production, including inflammatory responses (74, 75), cell proliferation (76, 77), induction of neuronal apoptosis during development (78), and release of neurotransmitters (79, 80). Thus, NOX function in microglia is important for health and normal physiology of the CNS, however, when excessively activated, it may also contribute to disease progression. A variety of CNS pathologies characterized by neuronal death seems to have a crucial participation of microglia-derived ROS, in particular, when activation of NOX enzymes is combined with generation of nitric oxide, resulting in extensive neuronal death via peroxynitrite production (81, 82). Excessive ROS generation by microglia contributes to the aggravation of neuronal damage after stroke or in neurodegenerative diseases (83, 81, 82), and plays a possible role in the development of psychiatric disorders (84, 85, 86). In addition, there is evidence for a role of microglial ROS generation in neurotoxicity associated with infections (e.g. Human Immunodeficiency Virus (87), Mycobacterium Tuberculosis (88), and LPS (lipopolysaccharide) (90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100) or with chemicals, such as paraquat (101, 102, 103), lindane and dieldrin (104, 105), diesel exhaust particles (106) or Zn2+ (107).
Astrocytes

Astrocytes are star-shaped glial cells which provide nutrients for neurons and regulate their activity. However, in pathological conditions, astrocytes can also contribute to inflammatory processes and neuronal cell death. Several NOX isoforms are expressed in astrocytes. Production of ROS by NOX enzymes plays a role in astrocyte signaling (108), survival (109) and production of proinflammatory mediators (75). Nonetheless, following toxic stimuli, activation of NOX enzymes in astrocytes can also induce damage of astrocytes (110, 111, 112, 113, 114, 115) or neurons (116, 117).

Oligodendrocytes

Oligodendrocytes are cells responsible for the production of myelin around neuron axons in the CNS. Although little is known about NOX expression in oligodendrocytes, they can in certain circumstances be very sensitive to damage by NOX-derived ROS (118, 119).
3. Role of NOX enzymes in central nervous system disorders

Several studies have analyzed the involvement of NOX enzymes in ROS overproduction underlying CNS diseases. In this chapter, after a brief introduction about each specific pathology, we first discuss the evidence for an involvement of NOX enzymes derived from patient studies, then data from animal models, and finally mechanistic and molecular insights from analyses of in vitro systems.

Neurodegenerative diseases

Alzheimer disease
Alzheimer disease (AD) induces cognitive dysfunctins and represents the most common single cause of dementia worldwide. The underlying mechanisms leading to the development of the disease are only partially known. Even for the small percentage of cases where genetic factors have been identified (mutations of the β-amyloid precursor protein (APP), presenilin 1 and 2), the sequence of pathological events is not fully understood (120). The typical microscopic hallmark of AD is the presence of intraneuronal neurofibrillary tangles and extra cellular senile plaques. The neurofibrillary tangles are a compact filamentous network formed by paired helical filaments composed of abnormal phosphorylated tau protein. The senile plaques are generated by a deposition of fibrils of the β-amyloid peptide (Aβ) (121). These abnormal protein deposits are associated with an accumulation of activated microglia and astrocytes, and profound synaptic and neuronal loss. Neuronal oxidative stress represents another commonly observed feature of AD (122), due in particular to glia activation (123).

Patient studies
Several studies indicate that NOX enzymes are involved in the pathomechanisms of AD (124). Activation of NOX2 in the brain of AD patients has been demonstrated, based on the translocation of NOX2 subunits (125). It appears that Aβ can cause NOX2-dependent ROS production, since the exposure of neutrophils and monocytes of CGD patients to Aβ-peptides did not induce H2O2 production (126). In addition, increased levels of mRNA transcripts of
NOX1 and NOX3 were also described in early stage brain tissue from AD patients (127). Thus, it appears that NOX2 is activated in AD patients, but there is also an up-regulation of other NOX enzymes, which might contribute to oxidative stress.

*Animal models*

Demonstrating the crucial role of NOX2 in AD development, NOX2-deficiency improved the outcome in a mouse model of AD. Mice that overexpress the Swedish mutation of APP (Tg2576, which leads to Aβ fragments accumulations) were crossed with NOX2-deficient mice. Absence of functional NOX2 was protective and prevented the negative effects of Aβ deposits. Neuronal oxidative stress was abrogated, and behavioral deficits improved in both young (3-4 months old) and aged (12-15 months old) Tg2576/NOX2-deficient mice (128, 129). Lack of NOX2 did not affect the accumulation of β-amyloid fragments, indicating that the NOX2-derived ROS are important player in pathological alterations induced by β-amyloid. Thus, the formation of senile plaques is not NOX-dependent, however the toxicity of the plaques is markedly amplified through NOX2-dependent ROS generation. In addition, a NOX2 inhibitory peptide, gp91ds-tat, reduced both oxidative stress and AD pathology in aged mice (129). This observation suggests that NOX inhibitors could act in an advanced state of the disease and, for this reason, they are possible candidates for AD therapy (130).

*In vitro models*

The role of NOX enzymes in AD pathology has been studied by using *in vitro* culture of microglia, astrocytes and neurons.

**Microglia**: Given that i) the presence of activated microglia around Aβ represents a major characteristic feature of neuro-inflammation in AD brains (131) and ii) ROS are released from activated microglia and contribute to neurotoxicity (81), the role of NOX2 in microglia has been investigated in different AD models *in vitro*. Exposure to Aβ fragments stimulates the assembly of NOX2 complex on the plasma membrane of microglia and consequently the production of ROS (132), which in turn causes functional alterations (133) or death (134) of neuronal cells. Similarly, using a co-culture system with human neuroblastoma cells overexpressing APP or mutated APP and microglia/macrophages, it was shown that neurons, releasing Aβ fragments, activate NOX2 in microglia, which produces oxidative stress and
leads to neuronal death (135). Also, the protective effect of melatonin in AD models was attributed to decreased ROS production following β -amyloid exposure due to inhibition of NOX2 complex formation in microglia (136). In addition to its neurotoxic effect, NOX2-dependent ROS production seems to influence microglial proliferation, which is stimulated by Aβ through the release of proinflammatory cytokines (e.g. TNF-α, IL-1β) (137). Different pathways involving NOX2 activation in microglia have been investigated: i) in an autocrine manner, ATP released from Aβ-stimulated microglia induces the production of ROS by NOX2 through activation of the purinergic receptor P2X7 (138, 139), ii) the interaction of Aβ with microglial cell surface receptor complex (140) leads to the tyrosine phosphorylation of Vav-GEF, a guanine nucleotide exchange factor for Rac1, thereby affecting the assembly of NOX2 (141), iii) β-amyloid causes NOX-dependent ROS generation, and subsequent oxidation-dependent activation of the chloride channel CLIC1. This oxidation is presumably due to the formation of an intrachain disulfide bond that promotes CLIC1 dimerization and activation (142). Sustained NOX activity itself in this model, however, also appears to depend on activity of the chloride channel, possibly providing a mechanisms for excessive ROS generation.

Astrocytes: An accumulation of astrocytes has been observed near amyloid plaques, and several references indicate that Aβ-induced NOX2 activation in astrocytes contributes to neurodegeneration (143-117). In addition, Aβ-peptides through NOX2-dependent ROS generation in astrocytes alters their membrane structure (144) and leads to depolarization of astrocyte mitochondria (117, 145). Both these factors might therefore contribute to AD pathology.

Neurons: Contributing to neurodegeneration, Aβ-peptides induce NOX2 activation not only in glia cells, but also directly in neurons (146, 147). In addition, the expression of particular mutated form of presenilin 1 and 2 (presenilin 2 N141L mutated or presenilin 1 with mutations in the C-terminal fragment) associated with familial forms of AD, caused directly neuronal death, which was abolished by apocynin (148, 149). Thus, NOX2 might also be directly expressed in neurons of AD patients and, thereby, contribute to oxidative stress and cell death.
In summary, there is strong evidence that oxidative stress in Alzheimer disease involves ROS generation by NOX enzymes, in particular NOX2. The precise mechanisms of action of NOX enzymes in AD, however, need further evaluation, and it is likely that, in addition to the well-established NOX2/microglia axis, there are other sites of NOX2 action.

**Parkinson disease**

Parkinson disease (PD) is a progressive neurodegenerative disorder, which primarily leads to motor dysfunctions due to loss of dopaminergic neurons in the substantia nigra and the consequent dopamine deficiency. With the exception of rare familial cases, the etiology of PD is still unclear, and both genetic and environmental factors have been implicated (150). Oxidative stress is thought to be an important pathogenetic factor for the degeneration of dopaminergic neurons in PD. Mitochondrial dysfunction has been proposed to be a major source of oxidative stress in PD (151), however, there is increasing evidence for a role of NOX enzymes.

**Patient studies**

Several studies have shown the presence of oxidative stress markers in samples of PD patients (152). In particular, increase in lipid peroxidation (153) and reduction of glutathione levels (154) were found to occur in substantia nigra of PD patients. However, to our knowledge, the role of NOX enzymes in patients with PD has not been investigated.

**Animal models**

Since the toxic compound MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and its derivative MPP⁺ (1-methyl-4-phenylpyridinium) cause effects similar to PD symptoms, in animals they are widely used to study PD (249). Translocation of p67phox was induced by MPTP in mouse brain and prevented by minocycline, an inhibitor of microglia activation (155). In addition, degeneration of dopaminergic neurons following administration of MPTP was attenuated by approximately 20% in NOX2-deficient mice as compared to wild type controls, suggesting the contribution of NOX2 in this process (156). In line with these findings, LPS-injections in the substantia nigra caused reduced death of dopaminergic neurons in NOX2-
deficient mice as compared with wild type animals (157). Thus, NOX2 activation could play a role in microglia-mediated loss of dopaminergic neurons in PD.

**In vitro models**

Most of the information pertaining to the role of NOX enzymes in PD pathogenesis emanates from *in vitro* studies. In particular, the involvement of NOX2 in microglia-dependent dopaminergic neurotoxicity has been consistently observed. Indeed, reactive microgliosis is a hallmark of PD brains (158). Exposure to rotenone (an herbicide), LPS, MPTP/MPP+, fMLP (formyl-methionyl-leucyl-phenylalanine), 6-OHDA (6-hydroxydopamine) and angiotensin II induce selective death of tyrosine hydroxylase (TH)-positive neurons in primary mesencephalic cultures. For the following reasons, it is thought that these compounds exert their toxicity at least in part through microglia NOX2 (159, 93, 160, 161, 162, 163): i) the toxicity toward dopaminergic neurons of these compounds was markedly enhanced in neurons/microglia mixed cultures as compared to neurons-enriched cultures ii) apocynin or NOX2-deficiency attenuated the deleterious effect of microglia on TH-positive neurons. In a similar manner, aggregated β-synuclein and substance P, two major components of protein aggregates in PD, activate NOX2 in microglia thereby contributing to dopaminergic neurodegeneration (164, 165). These results are corroborated by the analysis of the neuroprotective mechanisms of several compounds: dextromethorphan, sinomenine, FLZ (a squamosamide derivative), nimodipine, TGF- β1 (transforming growth factor-beta1) and pituitary adenylate cyclase-activating polypeptides (PACAP38, 27 and 4-6). Indeed, these compounds are thought to inhibit activation of microglial NOX2 (94, 95, 166, 167, 168, 100). However, microglia are probably not the primary cause of neuronal damage in PD, but rather a positive feed-back loop that amplifies neurotoxic stimuli. For example, MPP+-stressed neurons release mediators, such as matrix metalloproteinase-3, which in turn activates microglial NOX2, causing oxidative stress and enhanced neuronal death (169). On the other hand, the macrophage Ag complex-1 (MAC1), a microglial surface receptor, mediates microgliosis also by inducing the translocation of p47phox and NOX2-dependent ROS production (170).
The role of NOX enzymes in PD might not be limited to microglial NOX2. NOX1 may also be expressed in microglia and contribute to neurotoxicity (171). Also, a direct expression of NOX enzymes in dopaminergic neurons might play a role (172).

**Amyotrophic lateral sclerosis**

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease affecting motor neurons. First described by Jean-Martin Charcot in 1874, ALS is characterized by muscle weakness and atrophy that lead to paralysis and death usually within 5 years from disease onset. The majority of ALS cases are sporadic, but familial forms have been also identified (5-10%). Sporadic and familial forms seem to be characterized by similar neurotoxic mechanisms and, in particular, by oxidative stress (173). However, the primary cause of this pathology is largely unknown. Point mutations in the gene coding for the copper/zinc superoxide dismutase (SOD1) are associated with approximately 15-20% of familial forms of ALS, *i.e.* approximately 1% of total cases. It appears that the induction of the disease by SOD1 mutations is not due to a loss of SOD1 function, but rather to the neurotoxicity of the mutant protein (174).

**Patient studies**

Oxidative stress plays a central role in the development of ALS. Oxidative stress markers are elevated not only in cortex and spinal cord of ALS patients, but also in cerebrospinal fluid, plasma and sera (175). Expression of NOX2 at both messenger and protein level was increased in spinal cord of sporadic ALS patients. In particular, expression of NOX2 was specifically localized in microglial cells and led to ROS production and oxidative damage (176).

**Animal models**

The most widely used experimental model to study ALS is a mutant SOD1 transgenic mouse. This mouse develops motor neuron degeneration and neurological symptoms comparable to those observed in both sporadic and familial ALS patients (177). In this animal model, microglia activation contributes highly to neurodegeneration. Indeed, restricted expression of SOD1 mutant in astrocytes (178) or in motor neurons (179, 180) does not lead to motor neuron degeneration, while specific reduction of mutant SOD1 in microglia slows the course of the
disease and increases survival (181). Since oxidative damage in spinal cord and cerebral cortex is one of the main feature of ALS progression in SOD1 mutant mice (182), the involvement of NOX enzymes has been investigated in this model.

A crucial role of NOX2 has been demonstrated by two studies on SOD1 mutant mice crossed with NOX2-deficient mice. In both studies NOX2 deficiency increased lifespan, improved symptoms and decreased histological severity (183, 176). However, the magnitude of the effect was different. In one study, mean increase in lifespan was 13 days, while in the other it was around 100 days. It has been argued that differences in genetic background could account for these differences (183).

The involvement of NOX2 in the ALS mouse model was confirmed in other unrelated models: i) deletion of the prostaglandin receptor EP2 in aging SOD1 mutant mice improved motor strength and survival. The effects of this EP2 deletion included a reduction of NOX2 subunits p47phox, p22phox, p67phox and p40phox (184). ii) In the same manner, the lack of functional T cells or CD4+ T cells in SOD1 mutant mice leads to accelerated disease progression accompanied also by elevated NOX2 expression (185).

In addition to NOX2, NOX1 might also play a role in ALS pathogenesis. Indeed, NOX1 deficiency significantly increased lifespan in SOD1 mutant mice (183).

In contrast, pharmacological studies using the antioxidant/NOX inhibitor apocynin in the mouse ALS model produced contradictory results. In one study, the administration of high doses of apocynin (30-300 mg/Kg) increased survival and slowed ALS progression in SOD1 mutated mice (186). Conversely, a more recent analysis using similar apocynin concentrations did not indicate such effect (187). To our understanding, these contradictions do not raise doubts about the participation of NOX2 in ALS pathology, but rather demonstrate technical issues with the relatively non-specific inhibitor apocynin.

Thus, it appears that activation of NOX2, and to some extent also NOX1, is a relevant neurotoxic factor in ALS. There is strong evidence suggesting that microglial NOX2 is crucial, however experimental conditions do not allow discriminating the specific cellular type involved and other cellular sources of NOX enzymes (e.g. neurons) might also play a role (Figure 3).
**FIG. 3. Model for the involvement of NOX enzymes in ALS pathogenesis.** ALS is due to degeneration of motor neurons, which progressively causes muscle atrophy, paralysis, and death. SOD1 mutated protein (green triangle) causes damage in motor neurons, leading to generation of ROS by NOX2 and to release of mediators (1) that induce microglia activation (2). Activated microglia produce large amounts of ROS through NOX2 and amplify neuronal damage (3). Accumulation of ROS from NOX2 activation in both neurons and microglial cells leads to neuronal death (4) and aggravates ALS symptoms.

**In vitro models**

A recent study suggests that SOD1, interacting with the small GTPase Rac protein, might be involved in the regulation of NOX2 activity. For this reason, SOD1 mutations, by altering the correct binding with Rac, increase NOX2 activation (186). Even if microglia activation appears to be involved highly in ALS pathogenesis, it has been observed that SOD1-mutated astrocytes can also contribute to motor neuron loss, via activation of NOX2 (188).

As mentioned above, SOD1 mutations account for approximately only 1% of ALS cases. It may however be suspected that persistent NOX2 activation is a common down-stream pathway triggered by other possible mechanisms underlying ALS pathology. In particular, inflammatory processes also contribute to disease development (189). Li *et al.*, recently showed that in organotypic spinal cord slices, high dose of LPS induced NOX2-dependent death in motor neurons (190).
Cerebrovascular Diseases

Several NOX isoforms are highly expressed in the cerebral vasculature. For this reason, the role of NOX enzymes in cerebrovascular pathology, such as in cerebral stroke, has received wide attention. Stroke is the third leading cause of death and one of the major causes of disability worldwide. A stroke can be triggered either by the occlusion of a vessel in the brain (ischemic stroke) or by an intracranial hemorrhage (hemorrhagic stroke). Almost 85% of the strokes are ischemic, and the remaining is hemorrhagic. Symptoms may vary and depend on the brain area affected by the stroke, the extent of the insult and the vulnerability of neurons (200). One of the molecular events that follow stroke is the generation of ROS (201). In this context, several lines of evidence indicate an involvement of NOX enzymes in the mechanisms underlying the development of brain damage.

Ischemic Stroke

Ischemic stroke is caused by decreased blood supply in a defined brain region due to the occlusion of a vessel. This occlusion can be thrombotic (caused by blood clots formed locally) or embolic (caused by emboli arising from the heart or other part of the body). Atherosclerosis is one of the major risk factor for thrombotic stroke, whereas endocarditis and myocardial infarction are common causes of embolic stroke (200). Following ischemia, necrosis occurs in the infarcted area, while a series of complex molecular pathways are activated in the neighboring region, namely the penumbra. The penumbra is functionally impaired, but potentially salvageable (202). Thus, therapeutic concepts for the treatment of stroke should be designed to reduce the progression of brain damage and, hence, target the penumbra.

Patient studies

The pathogenesis of ischemic stroke in humans is thought to comprise genetic, environmental, and lifestyle factors. Genetic factors may increase the risk of developing a stroke (203) or affect the outcome in response to a given ischemic lesion (204). Among NOX subunits, p22phox presents the highest density of single nucleotide polymorphisms (SNP) (21). For this reason, investigations on the relationship between genetic variability of NOX enzymes and
cardiovascular diseases have focused on p22phox (205). However, the results are debatable. In two cases the T allele of the C242T polymorphism of p22phox was significantly associated with increased occurrence of cerebrovascular diseases (206, 207), but these data were not confirmed in other studies (208, 209). These discrepancies most likely reflect the fact that in a strongly polymorphic gene, such as p22phox, the effect of haplotypes (i.e. the constellation of SNPs in a given allele) rather than a single SNP should be studied.

**Animal models**

Permanent middle cerebral artery occlusion and transient middle cerebral artery occlusion followed by reperfusion are widely used in experimental animal models to investigate ischemic stroke. Following ischemia/reperfusion in adult mice, the absence of NOX2 significantly reduced infarct size and blood-brain barrier disruption (210, 211). However, the protective effect of NOX2-deficiency was not observed in newborn pups after hypoxia/ischemia (212). Based on bone marrow transplantation experiments, it has been suggested that, after stroke, NOX2 is a major source of ROS not only in peripheral leukocytes (invading the CNS with the reperfusion), but also directly in CNS cells (211). The expression of NOX2 after stroke has been observed in activated microglia of the penumbra region (213). However, NOX2 in astrocytes and neurons might also play a role. In addition to NOX2 expression, increased NOX4 mRNA level has been also measured in the cortex after stroke (214), in particular in neurons and in newly formed capillaries of the peri-infarct region (215). Several pharmacological studies have demonstrated that NOX enzymes are involved in the progression of brain damage after ischemic stroke. By using the non-selective electron transporter inhibitor DPI, superoxide production was reduced *ex vivo* in arteries of the penumbra area and of the controlateral part to the lesion (216). Also, the administration of the antioxidant/NOX inhibitor apocynin prior to ischemia exerted a protective effect (210, 217, 24, 25, 63). In particular, it has been suggested that apocynin decreases the activity of matrix metalloproteinase-9 (218), reducing the relative disruption of the blood-brain barrier and improving neurological outcome. It has been argued that the protective effect of apocynin can be only observed within a narrow range of concentrations and that it is lost at 5 mg/Kg (234).
However some studies have successfully used higher concentrations (210). Whether apocynin can be effective if administered after stroke remains to be tested. Other arguments indicating the deleterious effect of NOX-dependent ROS production in the development of post-ischemic brain lesions derive from study on:

i) Atorvastatin, which decreases NOX activity through reduction of Rac isoprenylation, and reduces brain damage after stroke (219, 210)

ii) Valsartan, an inhibitor of the angiotensin AT₁ receptor, which attenuates NADPH oxidase activity and superoxide production in the brain of salt-loaded stroke-prone hypertensive rats, and decreases neuronal apoptosis and inflammation (220).

iii) Normobaric hyperoxia treatment, which inhibits NOX2 expression and activity in cerebral microvessels after transient ischemia, and reduces matrix metalloproteinase-9 induction, blood-brain barrier damage, and cerebral edema (218),

iv) The protective effect of post-ischemic treatment with adrenomedullin - a CNS peptide hormone - which has also been associated with decreased NOX activity (221, 222).

However, NOX2 may not only be detrimental in the context of stroke. Indeed, ethanol preconditioning, which induces a mild oxidative stress through activation of NOX2, prevents excessive brain damage in experimental stroke (223).

*In vitro models*

Insights into the mechanisms underlying cellular death after stroke come from *in vitro* studies by using neuronal cultures exposed to oxygen/glucose deprivation and reoxygenation. It appears that NOX2 in neurons can contribute to ROS accumulation and neuronal death during the reoxygenation phase, whereas following anoxia the first source of ROS are mitochondria and xanthine oxidase (224).

**Hemorrhagic stroke**

Hemorrhagic strokes are often due to intracerebral or subarachnoid hemorrhage. Hypertension, bleeding disorders or amyloid angiopathy can cause intracerebral hemorrhage, whereas trauma or rupture of an aneurysm may lead to subarachnoid hemorrhage (200). Generally, the mortality rate is higher after hemorrhagic stroke than after cerebral ischemia. In
particular, the most severe and frequent complication of aneurysmal subarachnoid hemorrhage is cerebral vasospasm, due to altered vasoconstrictor and vasodilator mechanisms in cerebral vessels. These vasospasms lead to a high mortality and morbidity (225).

Patient studies

Signs of reduced antioxidant defense and of oxidative damage have been observed in cerebrospinal fluid, brain and serum of patients after subarachnoid and intracerebral hemorrhage (226, 227, 228, 229). In particular, it seems that oxidative stress could be responsible for development of vasospasms (230, 231). So far, patient oriented research on the role of NOX enzymes in ROS production following hemorrhagic stroke have not been performed. The only available study reports no association between p22phox polymorphisms and frequency of aneurysms (232).

Animal models

Studies on the role of NOX enzymes in intracerebral and subarachnoid hemorrhage using pharmacological inhibition or genetic deletion have produced conflicting results.

Rat model of subarachnoid hemorrhage: Published data have consistently shown a role of NOX enzymes in this model. Indeed, increased expression levels of NOX2, as well as decreased oxidative stress, vasospasms, and neurological deficits after treatment with the inhibitors, DPI or apocynin, were reported (233, 234, 235). It has also been argued that the diminished neuronal injury after treatment of subarachnoid hemorrhage with hyperbaric oxygen was due to reduced NOX2 expression (236, 237). However, since inhibitors are non-specific and no NOX2-deficient animals were tested, the evidence should, at this point, be considered as circumstantial.

Mouse model of subarachnoid hemorrhage: NOX2-deficient mice after subarachnoid hemorrhage failed to show a reduced mortality rate, brain water content or intensity of oxidative stress (238). Inhibitors have not been tested in this model, nor have mice deficient in other NOX enzymes.

Rat model of intracerebral hemorrhage: Treatment with apocynin did not improve the outcome of rats after intracerebral hemorrhage. Hemorrhage volume, neurological score and
brain edema were not reduced in rats injected with different doses (3-30mg/Kg) of apocynin (239).

**Mouse model of intracerebral hemorrhage:** After collagenase-induced intracerebral hemorrhage, NOX2-deficient mice displayed diminished bleeding, brain injury, neurological deficits and mortality as compared with wild type mice (240). An increased activity of NOX enzymes was also found in hypertensive mice with spontaneous intracerebral hemorrhage (241).

The question whether NOX enzymes are involved in the pathogenesis of hemorrhagic stroke remains open. In the context of an intracerebral hemorrhage, the protection of NOX2-deficient mice is of interest, and the absence of an effect of apocynin is only of relative weight. In the case of subarachnoid hemorrhage, the data is largely based on inhibitors and contradicted by the data from the NOX2-deficient mouse.

**Demyelinating diseases**

**Multiple sclerosis**

Multiple sclerosis (MS) is a demyelinating neurodegenerative disease that leads to severe chronic disability and gradually to death. It is a neurological disorder with an autoimmune component. It involves, however, other factors, including altered inflammatory response, neuronal loss and axonal injury processes (242). Since environment and genetics strongly influence disease outcome, disease progression may vary largely among patients (243). Several general lines of evolution of the disease have been described. It could be characterized by acute attacks (relapses) followed by remission (relapsing-remitting multiple sclerosis) or by a progressive neurological decline (244). Unfortunately no curative treatment exists, but partially effective therapies are currently available, which are generally based on anti-inflammatory treatment and immune modulation or suppression. The observations on the role of NOX enzymes in MS are puzzling. Indeed, NOX2 activity appears damaging in some studies, but protective in others. Although this is only partially understood, it is possible that
NOX2-dependent suppression of T cell activation at early disease stages is protective, whereas NOX2-dependent ROS release from microglia has a detrimental effect.

Patient studies
Evidence of the involvement of oxidative stress in the progression of MS has been reported in several studies on MS patients (e.g. (245, 246, 247)). Activation with PMA (phorbol-12-myristate-13-acetate) triggered higher ROS production in monocytes from MS patients than in controls (248), which might suggest a role of NOX2. Also, a diminished NOX-dependent ROS generation was measured in monocytes from interferon-γ treated patients (249). However, as discussed above, there are also patient data suggesting a protective effect of NOX2 in the context of MS. Indeed, a recent genetic study raised the possibility that the individual genomic pattern of p47phox is related to the age of disease onset in MS patients. The ratio between the two types of p47phox pseudogenes (one containing the deletion (ΔGT) and one not (GTGT)) was analysed. It was found that a ΔGT/GTGT ratio ≥ 2:1 is associated with lower oxidative burst and earlier disease onset as compared with a ΔGT/GTGT ratio ≤ 1:1 (250).

Animal models
Experimental autoimmune encephalomyelitis (EAE) is widely used in animals to model and investigate multiple sclerosis (251). The data on the effects of NOX2 activation in this model have given complex results. Depending on the size of the antigen that was injected to trigger EAE, p47phox deficient or p47phox mutant mice developed either a less severe or a more severe disease (252, 253). Also, in rats with a low ROS generating variant of p47phox experimental autoimmune encephalitis was aggravated (254).

In vitro models
Several observations indicate that NOX enzymes could be involved in mechanisms of myelin loss and inflammation mediated by macrophages and microglia. Phagocytosis of myelin by microglia/macrophages leads to NOX2-dependent ROS production (255, 256). However, it is not clear whether this increased ROS generation is detrimental and causes death of oligodendrocytes (118, 257), or whether it is neuroprotective because of decreased production of proinflammatory mediators (255).
Psychiatric disorders
Most research on psychiatric disorders has focused on abnormalities in different neurotransmitter systems. There is, however, increasing evidence for a role of oxidative stress in the pathogenesis of mental diseases, in particular schizophrenia, bipolar disorder, anxiety or autism. It seems that oxidative stress is a common characteristic of these pathologies (258). Thus, the question to which extent NOX enzymes are involved in the pathogenesis of mental diseases is becoming increasingly relevant. In particular, recent studies have suggested a possible role for NOX enzymes in the pathophysiology of schizophrenia and anxiety.

Schizophrenia and drug-induced psychosis
Approximately 1% of the world’s population is affected by schizophrenia (259). It usually appears in the late second and third decades of life and manifests with altered mental functions (hallucinations, disturbance in cognitive functions, attention and memory) and abnormal behavior (loss of motivation or excessive emotionality). Both genetic and environmental factors have been strongly correlated with the development of schizophrenia. Affected individuals suffer from a lifetime of disability and about 10% will eventually commit suicide (260). Presently, therapeutic options for schizophrenia are limited and, in general, consist of drugs that act mainly on symptoms. Moreover, they have significant adverse effects, and have to be taken chronically.

Patient studies
Evidence of the involvement of oxidative stress in the mechanisms of schizophrenia was first proposed in 1954, and subsequent studies have confirmed the contribution of ROS in mechanisms underlying this mental disease. Specifically, analyses on schizophrenic patients have revealed decreased antioxidant defense, such as glutathione (261), and signs of oxidative membrane lipid damage (262). Microglia activation has been detected in brain regions of schizophrenic patients (263, 85, 264), raising the possibility that microglial NOX enzymes might play a role. In addition, an increased superoxide production in neutrophils from schizophrenic patients was observed as compared with controls (265).
Animal models

Certain drugs, in particular ketamine and phencyclidine, may cause schizophrenia-like symptoms, in humans and in animals (266). Recent reports have investigated the role of NOX enzymes in ketamine-induced psychosis. In this experimental system, such chronic administration of subanesthetic doses of ketamine led to oxidative stress and decreased the number of parvalbumin-expressing GABAergic interneurons, causing excessive activation of pyramidal neurons and related psychosis (23). Given that i) ketamine augmented NOX2 expression in the mouse brain, and that ii) both apocynin pre-treatment and NOX2-deficiency prevented ROS generation and the decrease of parvalbumin-expressing interneurons, NOX2 activation was indicated as a major contributor to the pathogenesis of ketamine-induced psychosis and possibly also to schizophrenia (23, 72). In this context, it was suggested that enhanced neuronal production of interleukin-6 is responsible for NOX2 upregulation and/or activation in the brain after ketamine injections (72) (Figure 4).

**FIG. 4. Involvement of NOX enzymes in ketamine-induced psychoses.** (A) Pyramidal neurons (blue) activate GABAergic parvalbumin interneurons (green) via NMDA receptors. Activated parvalbumin interneurons inhibit pyramidal neurons, providing a negative-feedback reaction. (B) Ketamine blocks NMDA-receptor signaling on parvalbumin-positive neurons. Thus, they lose their inhibitory tone on pyramidal neurons. (C) Enhanced NOX2 expression and activation in GABAergic neurons leads to decrease of parvalbumin, possibly through neuronal production of interleukin-6 (IL-6). This process contributes to ketamine-induced psychosis.
In vitro models
Using isolated primary neurons culture, it was observed that exposure to ketamine leads to oxidative stress and parvalbumin-expressing neurons decrease. Since apocynin reversed ketamine effects, the role of NOX enzymes was suggested (23). As observed in vivo, interleukin-6 mediated NOX2 activation also in purified primary neurons (72).

Anxiety
Anxiety is basically a physiological reaction that can affect anybody. It is characterized by behavioral and physiological alterations, such as accelerated heart rate, increased breathing, and muscles tension. However, anxiety symptoms can become pathological when they manifest without any obvious stimuli and start to interfere with normal life. In those cases, anxiety represents the predominant feature of a more severe mental disorder, which includes phobia, social anxiety, post-traumatic stress or panic disorder. Genetic and environmental factors influence moderately the development of anxiety as compared with other mental diseases. Nevertheless, they can affect cerebral maturation during childhood and cause anxiety-related behavior in adult age (267). Neurochemical alterations and anatomical structures related to anxiety symptoms are not yet completely clear, but increasing evidence suggest a role of oxidative damage in the pathogenesis of anxiety.

Patient studies
Several data from epidemiological studies suggest that anxiety-related pathologies are linked to altered antioxidant defense and increased oxidative lipid damage (268, 269). However, no information on the involvement of NOX enzymes in patients with pathological anxiety could be retrieved.

Animal and in vitro models
In mice, ROS contribute to anxiety-related behavior (270) and signs of oxidative stress are found not only in the brain (271), but also in peripheral blood cells (272) of animals anxious behavior. One report suggests that NOX enzymes activation could induce anxiety symptoms. Oxidative stress induced with BSO (L-buthionine-(S,R)-sulfoximine) caused anxiety-related behavior in animals. These anxiety symptoms were reversed by apocynin. In addition, the
expression of NOX2 and its subunits was increased by BSO in brain areas involved in the
development of anxiety (hypothalamus and amygdala) as well as in primary cortical neurons
(273).
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THIRD PART
1. DESCRIPTION OF THE STUDY: ROLE OF NOX2 IN THE PATHOGENESIS OF SCHIZOPHRENIA

Mechanisms and pathways underlying oxidative stress need to be better understood for the development of new therapeutic approaches targeting ROS in psychotic disorders. Therefore, the objective of this study was to investigate the role of the ROS generating enzyme NOX2 in animal models of schizophrenia. Two recent reports have suggested the involvement of NOX2 in schizophrenia pathogenesis, and it was shown that repeated administration of subanesthetic doses of ketamine increases NOX2 expression in the mouse brain and pharmacological inhibition or genetic deletion of NOX2 abolishes ROS generation induced by ketamine as well as the loss of expression of parvalbumin and gamma-aminobutyric acid-producing enzyme (GAD67) in GABAergic interneurons. In our study, we wanted to analyse whether NOX2 activation influences the development of schizophrenia-like symptoms using two different experimental models i) social isolation rearing of rats and ii) subanesthetic administration of ketamine in mice. As mentioned before, the first model reproduces the neurodevelopmental aspects of schizophrenia, and allows analysing the long-term effect of social isolation during a period crucial for the correct formation of the brain. In contrast, the pharmacological model allows a rapid induction of schizophrenia-like symptoms. In both cases, we have observed that NOX2-dependent ROS formation plays a major role in the development of neurochemical and behavioural alterations. In fact, after seven weeks of social isolation, the expression of NOX2 was dramatically increased in specific brain regions, including the nucleus accumbens and the prefrontal cortex. This augmentation was associated with signs of oxidative stress in the same brain regions. Moreover, an increase in immunoreactive microglia suggested that oxidative stress could be in part due to NOX2 activation in microglia. We have also demonstrated that the pharmacological treatment with the NOX inhibitor/antioxidant apocynin prevented behavioral and histopathological alterations caused by social isolation. In the second model, behavioral dysfunctions induced by ketamine were completely abrogated in NOX2-deficient animals. Similarly to the social isolation model, the increase of oxidative stress markers in brain regions involved in ketamine response were absent in mice lacking NOX2. By analyzing
glutamate and dopamine levels with cerebral microdialysis technique, we have also observed that ketamine caused their increased extracellular release in wild-type mice, while both neurotransmitter concentrations remained at basal levels in NOX2-deficient mice. Taken together, these results demonstrate that NOX2 is a major source of oxidative damage in two different animal models of schizophrenia. In particular, NOX2-derived ROS are regulating glutamate and dopamine release following ketamine injection. Also, NOX2 inhibition can reverse behavioural symptoms and neurochemical alterations. These studies are described in details in two research articles reported hereafter: the first describes the results obtained by using the social isolation model in the rat, while the second reports the different effects of subanesthetich doses of ketamine in wildtype and NOX2-deficient mice.
2. RESEARCH ARTICLE:

Involvement of NOX2 in the Development of Behavioral and Pathologic Alterations in Isolated Rats
Involvement of NOX2 in the Development of Behavioral and Pathologic Alterations in Isolated Rats

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Background: Social stress leads to oxidative stress in the central nervous system, contributing to the development of mental disorders. Loss of parvalbumin in interneurons is an important feature of these diseases. We studied the role of the superoxide-producing nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) in rats exposed to social isolation.

Methods: Male rats were kept for 7 weeks in group or in social isolation (n = 6–10 per group). Behavioral tests, immunohistochemistry, and analysis of NOX2 expression were performed at the end of social isolation. Apocynin was given in the drinking water (5 mg/kg/day).

Results: NOX2 was below detection level in the brains of control animals, whereas it was highly expressed in isolated rats, particularly in the nucleus accumbens and prefrontal cortex. Indirect markers of oxidative stress (oxidized nucleic acid 8-hydroxy-2'-deoxyguanosine, redox-sensitive transcription factor c-fos, and hypoxia-inducible factor-1α) were increased after social isolation in brain areas with high NOX2 expression. An increase in immunoreactive microglia suggested that oxidative stress could be in part due to NOX2 activation in microglia.

In response to social isolation, rats showed increased locomotor activity, decreased discrimination, signs of oxidative stress in neurons, and loss of parvalbumin-immunoreactivity. Treatment of isolated rats with the antioxidant/NOX inhibitor apocynin prevented the behavioral and histopathological alterations induced by social isolation.

Conclusions: Our data suggest that NOX2-derived oxidative stress is involved in loss of parvalbumin immunoreactivity and development of behavioral alterations after social isolation. These results provide a molecular mechanism for the coupling between social stress and brain oxidative stress, as well as potential new therapeutic avenues.

Key Words: Apocynin, GABAergic, interneurons, NADPH oxidase, oxidative stress, parvalbumin

There is compelling epidemiologic evidence that social stress leads to oxidative stress and neuronal reorganization in the central nervous system (CNS). Prolonged oxidative injury is involved in the development of mental diseases (1), in particular, of schizophrenia-like disorders. Many hypotheses exist on the pathogenesis of these brain dysfunctions (2). Among them, a change of phenotype of gamma-aminobutyric acid (GABA)ergic interneurons, including loss of parvalbumin expression, has been proposed (3,4).

In addition, prolonged exposure to high doses of N-methyl-D-aspartate (NMDA) receptor antagonists (such as ketamine and phencyclidine), which has been demonstrated to provoke psychotic symptoms in humans and behavioral alterations in rodents, also induces a decrease in parvalbumin protein expression (5–7). It has recently been suggested that the loss of parvalbumin expression and GABAergic function may be a central feature in the pathogenesis of schizophrenia (8).

The nicotinamide adenosine dinucleotide phosphate (NADPH) oxidases (NOX) are proteins that transfer electrons across biological membranes to produce superoxide. This family includes seven members (NOX1-5, DUOX1/2), with distinct tissue distribution and mechanisms of activation (9). Although their role in peripheral organs is generally well documented, their functions in the brain are poorly known. In neurons, NADPH oxidase 2 (NOX2) enzyme is thought to be involved in cell fate and modulation of neuronal activity (10). From a pathologic point of view, NOX enzymes are involved in the increase of oxidative stress seen in a variety of brain disorders, from psychiatric to neurodegenerative diseases (11). To confirm the role of NOX enzymes in the development of these brain pathologies, compounds that inhibit the activity of NOX proteins have been used in animal models (12). Among the proposed NOX inhibitors, apocynin (4-hydroxy-3-methoxy-acetophenone) has been shown to have multiple biological actions (13) such as anti-inflammatory and antioxidant effects in cell and animal models (14,15). Even if apocynin cannot be considered a specific NOX inhibitor, recent studies have shown that it is effective at reducing NOX-dependent superoxide and oxidative stress in rodents (16).

Progress in the understanding of the mechanistic link between oxidative stress and psychiatric diseases requires animal models. In a mouse model of ketamine-induced psychosis, a role for NOX2-derived superoxide production in the loss of phenotype of parvalbumin-positive interneurons has been shown (8,17).

The social isolation rearing of young adult rats (18,19) provides a nonpharmacologic method of inducing long-term alterations reminiscent of several symptoms seen in schizophrenic patients (20), including hyperreactivity to novel environments and cognitive impairment (21,22). Importantly, social isolation also induces deficits in parvalbumin-positive interneurons in the brain (23),

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The main aim of this study was to evaluate the possible role of NOX2 in the development of behavioral and pathological alteration induced by social isolation. We demonstrate that social isolation rearing induces NOX2 expression, thereby causing an increase in oxidative stress and microglia and a decrease of parvalbumin immunoreactivity. Apocynin administration prevents this decrease and the behavioral alterations induced by social isolation.

Methods and Materials

Animals

An equal number of adult male and female Wistar rats (Harlan, S. Pietro al Natisone, Udine, Italy) were used to obtain litters. A total number of 17 dams provided offspring for inclusion in the study. All animals were housed at a constant room temperature (22°C ± 3°C) and relative humidity (55% ± 5%) under a 12-hour light/dark cycle (lights on from 7:00 AM to 7:00 PM). Food and water were freely available. All efforts were made to minimize the number of animals used and their suffering in conformity with ethical guidelines and national and international laws (DL Number 116, G. U, suppl. 40, 18 Febbraio 1992, Circolare Number 8, GU, Luglio 14, 1994; EEC Council Directive 86/609, OJ L 358, December 1, 2012, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Social Isolation Protocol

The social isolation procedure was performed on male rats following the protocol described by Leng et al. (19). Litters were culled to have six males and two females in each litter. At weaning (postnatal Day 21), male pups were separated from their mothers and reared either in social isolation (isolated: one rat per cage) or in social groups (control: four or five rats per cage). To avoid a litter effect, one subject was put in the group of controls and one subject in the isolation condition. Details on labeling and enrollment of the animals in the study are described in Supplement 1. All animals were reared in Plexiglas cages (48.0 × 27.0 × 20.0 cm for the isolated; 59.0 × 38.5 × 20.0 cm for the controls) and disturbed only for cleaning purposes (once a week for the isolated animals and twice a week for control animals). Isolated and control rats were housed in the same room so that the isolated rats maintained visual, auditory, and olfactory contact with their mothers and reared either in social isolation (isolated: one rat per cage) or in social groups (control: four or five rats per cage). Water drinking was measured in drinking water intakes in the 24-hour test; similar results were obtained at later time points (data not shown).

Reverse Transcriptase Polymerase Chain Reaction

Total brain RNA was isolated using RNasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Residual genomic DNA was removed using RNase-Free DNase set (Qiagen). One microgram total RNA was reverse transcribed using the Superscript II Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Polymerase chain reactions (PCR) were performed at 35 cycles for target genes and at 30 cycles for the housekeeping gene β-actin. PCR conditions, primers, and product size have been previously described (26).

Western Blotting

Western blotting was performed according to the standard procedure using polyclonal antibodies for NOX2 (1:1000, Bio-sciences Pharmigen, Erembodegem, Belgium), parvalbumin (1:4000, kindly provided by Professor Do Kim, Centre Hospitalier Universitaire Vaudois, Lausanne), hypoxia-inducible factor-1 alpha (HIF-1α; 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), ionized calcium binding adapter molecule 1 (IBA-1, 1:1000, Dako, Glostrup, Denmark) and β-actin antibody (1:4000, Sigma-Aldrich). Fifty micrograms of proteins per lane were diluted in loading buffer and denatured at 70°C for 10 min. Optical densities of the bands were measured using ImageJ software (http://rsb.info.nih.gov/ij/) and normalized with β-actin.

Immunohistochemistry

At the end of social isolation period, animals were anesthetized and their brains fixed through intracardiac perfusion with paraformaldehyde 4% in phosphate buffered saline (PBS; pH = 7.35). Brains were removed, embedded in paraffin, cut in 10-μm-thick sections, and mounted on glass slides. Brain sections were deparaffinized through graded alcohols, subjected to heat-induced epitope retrieval for 15 min in 0.1 mol/l citrate buffer (pH 6.0), and incubated overnight at 4°C in PBS-3% Triton-X100 buffer containing antibodies raised against one, or a combination for double immunostaining, of the following: glial fibrillary acidic protein (GFAP; 1: 2000, Millipore, Billerica, MA), IBA-1, (1:5000, Dako), 8-hydroxy-2′-deoxyguanosine (8-OHdG, 1:10, JaIC, Shizuoka, Japan), parvalbumin (1:4000), c-fos (1:100, Sigma-Aldrich). For 3,3′-diaminobenzidinetetrahydrochloride hydrate (DAB) immunohistochemistry, sections were then incubated for 1 hour at room temperature with specific biotinylated secondary antibody (1:100, Vector Laboratories, Burlingame, California) and, after several washes in PBS, for 1 hour in Horseradish peroxidase-avidin/biotin complex solution (1:100, Vector Laboratories). Horseradish peroxidase was visualized using 3,3′-diaminobenzidinetetrahydrochloride hydrate (DAB, Sigma-Aldrich) and H2O2. Counterstaining with cresyl violet was performed to visualize cell nuclei.

For indirect immunofluorescence detection, brain sections were incubated with specific fluorescent-labeled secondary antibody (1:1000, Alexa Fluor 488 or 555). After several washes in PBS, brain sections were counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, Karlsruhe, Germany) before mounting with FluorSave (Calbiochem, Mississago, Ontario, Canada). Technical controls were performed for each experiment by omitting the first antibody and performing the other steps of the procedure (data not shown).

Behavioral Tests

Open Field Test. The open field test was carried out in 60 × 40–cm arena surrounded, by 50-cm-high brown plywood walls. The floor of the arena was divided with black lines into 12 equal rectangles. After a 1-week period of habituation, animals were initially placed in the same corner and then left to move freely in the arena for 20 min. Four intervals of 5 min were analyzed (27). We manually measured as spontaneous locomotor activity the total of horizontal (number of crossings of the black lines) and vertical (number of rearings) displacements performed during the test (28). Results refer to the first 5 min of the test; similar results were obtained at later time points (data not shown).

Novel Object Recognition (NOR) Test. The NOR test was performed in a Perspex box (39 cm × 23.5 × 24.5 cm) in
constant white light, between 9:00 AM and 2:00 PM. The method described by Ennaceur and Delacour in 1988 (29) and adapted by King et al. in 2004 (30) was followed. The test comprised two trials of 3 min with an intertrial time of 1 min (31,32). Briefly, in the familiarization trial, rats were left to explore two identical objects: (8 cm high × 5 cm diameter plastic bottles covered with a white tape). In the choice trial, the familiar object and a novel object (the same plastic bottle covered with white tape, but with three horizontal black strips) were presented to the same animals. The position of each bottle was inverted and located 5 cm from the side and 10 cm from the end wall in opposite corners of the box. Before the beginning of the experiment and between first and second trial, the objects were cleaned with 20% v/v ethanol to remove any olfactory cues, and the arena was cleared of rat feces. Exploration of the objects (sniffing, touching, and having moving vibrissae while directing the nose toward the object at a distance of 1 cm) was recorded manually by the investigator who was blind to the experimental identity of the tested animal. Sitting on, leaning against, and chewing the objects were not considered as exploration. The discrimination index was calculated using the following formula: \((N - F)/(N + F)\) \((N = \text{times spent in exploration of the novel object during the choice trial}; F = \text{times spent in exploration of the familiar object in the choice trial})\) (33).

Details on exploration times during test trials are described in Tables 1 and 2 in Supplement 1.

**Data Analysis**

Data were analyzed using Sigma Stat 3.1 software. The statistical tests (with F values and degrees of freedom when appropriate) are indicated in the figure legends. For all tests, a p value inferior to .05 was considered statistically significant. Results are expressed as means ± standard error (SEM).

**Results**

**Induction of NOX2 Expression in Rat Brain After Social Isolation**

We performed reverse transcriptase (RT)-PCR to assess the effect of social isolation on NOX2 gene expression in specific areas of rat brain: amygdala (AMY), hippocampus (HIPP), nucleus accumbens (NACC), prefrontal cortex (PFC), and striatum (STR). Whereas NOX2 mRNA was below detection levels in the five selected brain areas of control rats \((n = 5)\), NOX2 transcripts in isolated rats \((n = 5)\) were detected in four of these five brain regions (AMY, HIPP, NACC, and PFC, but not in STR). The increase in NOX2 gene expression was particularly important in NACC of isolated rats (Figure 1A).

We focused our interest on NACC and PFC because social isolation affects them in terms of neurochemistry, neuronal organization, and stress-induced injury (34,35). To verify that the subunits necessary for NOX2 activity were present (9), RT-PCR experiments were performed for \(p67^{phox}\), \(p47^{phox}\), \(p40^{phox}\), and \(p22^{phox}\) in control \((n = 5)\) and in isolated rats \((n = 5)\). RT-PCR results showed that although in the NACC and PFC of control animals NOX2 subunits were below detection levels, isolated rats presented an important increase in the expression of NOX2 subunits mRNA (Figure 1B).

To verify the translation of NOX2 mRNA, the expression of NOX2 protein was evaluated with Western blotting. In the NACC and PFC of control rats \((n = 5)\), NOX2 protein was not detected, whereas the expression of NOX2 protein was significantly increased after social isolation \((n = 5)\; \text{Figure 1C}\). In the PFC, NOX2 protein was weakly detected in control rats \((n = 5)\) and, as for NACC, social isolation induced an important increase of NOX2 protein \((n = 5)\; \text{Figure 1D}\). These findings indicate that social isolation induces the expression of NOX2 enzyme at mRNA and protein levels. In addition, an increase in NOX2 enzyme subunits was observed.

**Figure 1.** Effect of social isolation on NOX2 expression in selected areas of the rat brain. (A) Reverse transcriptase polymerase chain reaction (RT-PCR) for NOX2 and actin mRNA in selected rat brain areas: AM, amygdala; HIPP, hippocampus; NACC, nucleus accumbens; PFC, prefrontal cortex; STR, striatum. CTRL, control rats \((n = 5)\); ISO, isolated rats \((n = 5)\). (B) RT-PCR for NOX2 subunits \((p67^{phox}, p47^{phox}, p40^{phox}, p22^{phox})\) and actin mRNA in the NACC and PFC of CTRL \((n = 5)\) and ISO \((n = 5)\). (C) Western blotting for NOX2 and actin proteins in the NACC of CTRL \((n = 5)\) and ISO \((n = 5)\), with optical density of NOX2 protein bands normalized to the actin protein values. Statistical analysis: Student’s t test, \(* * * p < .001\). (D) Western blotting for NOX2 and actin proteins in the PFC of CTRL \((n = 5)\) and ISO \((n = 5)\), with optical density of NOX2 protein bands normalized to the actin protein values. Statistical analysis: Student’s t test, \(* * * p < .001\).
Increase of Oxidative Stress in the Brain After Social Isolation

To verify whether the increase in NOX2 enzyme was associated with signs of oxidative stress in the brain of isolated rats, immunohistochemical detection was performed on PFC and NACC of control (n = 4) and isolated rats (n = 4), using antibody raised against 8-OHdG, one of the most abundant marker of oxidative stress to DNA (36). Compared with control animals, there was a significant increase of 8-OHdG immunostaining in PFC and NACC of isolated rats (Figure 2A–2D). In addition, as indirect markers of oxidative stress, we evaluated the expression of the immediate early gene and redox-sensitive transcription factor c-fos and of the HIF-1α protein, which is known to be upregulated under oxidative stress condition (9). Immunohistochemistry showed an increase of c-fos immunoreactive cells number in the PFC and in the NACC of isolated rats (n = 4) compared with control rats (n = 4) (Figure 2I–L). An increase of HIF-1α protein was detected by Western blotting in the NACC and PFC of isolated rats (n = 6) compared with control animals (n = 6). This increase was greater in the NACC than in the PFC (Figure 2M,N). Conspicuously, there was no difference in HIF-1α protein level between the STR of control (n = 6) and isolated rats (n = 6), where NOX2 mRNA was not detected (Figure 2O).

Detection of Oxidative Stress in Glial Cells and Neurons

To determine which brain cell types affected by oxidative stress after social isolation, immunohistochemistry was performed in control (n = 4) and isolated rats (n = 4) for the astrocyte marker GFAP, the microglial marker IBA-1, the GABAergic interneurons marker parvalbumin, and 8-OHdG. Immunostaining showed that social isolation apparently did not affect GFAP immunoreactive staining (data not shown), but it induced an increase of the IBA-1 immunoreactive staining in the PFC, NACC, and forceps minor (Figure 2 in Supplement 1). This increase was confirmed by Western blotting (n = 6; Figure 3).

Oxidative Stress, Loss of Parvalbumin Immunoreactivity in Interneurons, and Behavioral Alterations Induced by Social Isolation Are Prevented by Apocynin

To detect signs of oxidative stress in microglial cells of isolated rats, double immunohistochemistry was performed for IBA-1 and 8-OHdG (n = 4) in the PFC and NACC: most microglial cells were stained for 8-OHdG (Figure 2A–2L in Supplement 1). In addition, nucleic acid oxidation was broadly observed in neurons, including parvalbumin-immunoreactive interneurons, of the PFC and NACC of social isolated rats, whereas little 8-OHdG immunoreactivity was detected in control brains (Figure 2M–2X in Supplement 1).

To test whether the signs of oxidative stress detected in the brain of isolated rats could be prevented pharmacologically, the antioxidant/NOX inhibitor apocynin was added to the drinking water of isolated rats during the period of social isolation (n = 10 per group). Immunohistochemistry for 8-OHdG and c-fos performed in the PFC and NACC of isolated rats (n = 4) showed that apocynin prevented the development of the signs of oxidative stress (Figure 3 in Supplement 1). In addition, Western blotting experiments showed a decrease in HIF-1α protein expression in apocynin-treated isolated rats compared with nontreated isolated rats (n = 6; Figure 4).
We next investigated the relationship between NOX2-dependent oxidative stress and the decrease in parvalbumin-immunoreactivity. For this purpose, we performed parvalbumin immunohistochemistry in the PFC and NACC of control and isolated rats \( (n/H_11005/4) \). Results showed that, compared with control, social isolation determined a decrease in parvalbumin immunoreactivity in both the PFC (Figure 5A–5D) and NACC of isolated rats (Figure 5I–5L). However, cresyl-violet-stained sections of the same brain regions showed no detectable differences between experimental groups (Figure 5E–5H and 5M–5P). The administration of apocynin during the period of social isolation prevented the loss of parvalbumin immunoreactivity in PFC and NACC as assessed by immunohistochemistry (Figure 5A–5D and 5I–5L) and Western blotting (\( n/H_11005/6 \) per group; Figure 5Q and 5R). These findings suggest a possible role of NOX-related oxidative stress in the loss of parvalbumin immunoreactivity induced by rearing in social isolation.

**Figure 3.** Increase of ionized calcium binding adapter molecule 1 (IBA-1) protein after social isolation. (A) Western blotting for IBA-1 protein in the prefrontal cortex of control (CTRL; \( n = 6 \)) and isolated (ISO; \( n = 6 \)) rats, with optical density of IBA-1 protein bands normalized to the actin protein values. Statistical analysis: Student’s \( t \) test, \( * p < .05 \). (B) Western blotting for IBA-1 protein in the nucleus accumbens of CTRL (\( n = 6 \)) and ISO (\( n = 6 \)) rats, with optical density of IBA-1 protein bands normalized to the actin protein values. Statistical analysis: Student’s \( t \) test, \( ** p < .01 \).

**Figure 4.** Effect of apocynin treatment on hypoxia-inducible factor-1 alpha (HIF-1\( \alpha \)) protein in the brain of isolated animals. (A) Western blotting for HIF-1\( \alpha \) protein in the prefrontal cortex (PFC) of isolated rats not treated and treated with apocynin (\( n = 6 \) per group) with optical density of HIF-1\( \alpha \) protein bands normalized to the actin protein values. Statistical analysis: Student’s \( t \) test, \( ** p < .01 \). (B) Western blotting for HIF-1\( \alpha \) protein in the nucleus accumbens (NACC) of isolated rats not treated and treated with apocynin (\( n = 6 \) per group) with optical density quantification of HIF-1\( \alpha \) protein bands normalized to the actin protein values. Statistical analysis: Student’s \( t \) test, \( ** p < .01 \).
To correlate molecular changes with behavioral observations, the open field and NOR tests were performed (n = 10 per group), with a focus on behavioral functions that are physiologically regulated by NACC and PFC, such as spontaneous locomotor activity and novel object discrimination (37). Behavioral tests showed an increase in spontaneous locomotor activity (Figure 6A) and a decrease in the discrimination index (Figure 6B) in isolated rats compared with controls. Seven weeks of apocynin treatment prevented these behavioral alterations (Figure 6A and 6B).

**Discussion**

In this study, we investigated the relationship among social-isolation-induced brain alterations, NOX2, and oxidative stress. We demonstrate that social isolation of young rats leads to an upregulation of NOX2 and all subunits (p47phox, p22phox, p67phox, p40phox) required for the activation of the enzyme. The upregulation of NOX2 is accompanied by signs of brain oxidative stress, increased microglia immunostaining, decrease of parvalbumin-immunoreactivity in neurons and behavioral alterations. The antioxidant/NOX inhibitor apocynin prevented signs of oxidative stress in the brain, the decrease of parvalbumin immunoreactivity, and behavioral alterations.

The fact that social stress leads to oxidative stress in the brain has been shown in rodent models (38,39) and in humans (1). There is good evidence that CNS oxidative stress is associated to anxiety; in particular, this is supported by the observations that chemical compounds that induced brain oxidative stress, such as L-buthionine-(S,R)- sulfoximine (BSO), lead to anxiety in rodents (40) and that long-term treatment with antioxidants, such as vitamin E (41), decreases anxiety. The molecular mechanism through which social stress induces oxidative stress is hitherto poorly understood. Therefore, upregulation of NOX2 seen in specific regions of the brain is of particular interest because it is an important source of reactive oxygen species (ROS) in the CNS. To the best of our knowledge, this study is the first indication that NOX enzymes—in particular, NOX2—might be involved in social-stress-induced oxidative stress.

There is increasing evidence that prolonged social stress leads to development of psychiatric disorders in humans (1). Similarly, as seen for the relationship between acute social stress and anxiety, long-term oxidative stress has been suggested to contribute...
factor (51) or tryptophan hydroxylase (52). One report shows that an oxidative stress inducer compound, 2,4,6-trinitrotoluene, causes the upregulation of neuronal nitric oxide synthase gene in rat cerebellum primary neuronal cells (53). However, few data actually exist on the molecular mechanisms involved in the upregulation of these genes. Here, we suggest an upregulation of the superoxide-generating enzyme NOX2 in the nucleus accumbens and in the prefrontal cortex as a likely source of oxidative stress and of subsequent pathological alterations in response to social isolation.

Because NOX2 is highly expressed in microglia (54), it is arguable that the increased microglial immunoreactivity observed in our study contributes to the increase in NOX2 and subsequent oxidative stress. Along these lines, we showed that an important percentage of microglial cells express signs of oxidative stress. Microglia has been implicated in the pathogenesis of schizophrenia (55,56) and a recent study reported microglia activation in patients with recent-onset schizophrenia (57). However, microglia is not the only source of CNS NOX2; it may also be expressed in neurons where, when activated, it may contribute to proinflammatory signaling or neuronal cell death (11). Thus, it cannot be excluded that the initial NOX2-dependent oxidative stress is generated in neurons and that microglia activation is a secondary inflammatory response.

Is NOX2 the only relevant source of ROS in response to social isolation? It would be premature to draw such a conclusion. There are other relevant sources of ROS in the brain such as mitochondria (58) and xanthine oxidase (59), among others (60). Also, other NOX enzymes are found in the CNS (11) and could be involved.

Another argument that, in the rat social isolation model, oxidative stress might be generated directly within neurons comes from our observation that neurons, including parvalbumin-immunoreactive interneurons, of isolated rats show signs of oxidative stress. However, further studies are necessary to distinguish the latter possibility from the hypothesis that oxidative stress detected in neurons could be due to diffusion of ROS (in particular, the nonpolar H₂O₂) from microglia to neurons. Irrespective of its precise source, the oxidative stress in parvalbumin-immunoreactive neurons from isolated rats observed in our study is significant for the pathogenesis of behavioral and pathologic alterations induced by social isolation. Indeed, the loss of parvalbumin expression in interneurons is a central feature of the pathophysiology of mental disorders (61), and it has been observed in the prefrontal cortex of schizophrenic patients (62). Also, in a mouse model of ketamine-induced psychosis, NOX2 was thought to be implicated in this loss (8,17).

Apocynin is considered an antioxidant compound because it can act as a ROS scavenger (63). However, under certain circumstances, it can block the activity of NOX enzymes, possibly by interfering with the assembly of the cytosolic NOX components with the membrane (13). It has been demonstrated that apocynin can penetrate the intact blood-brain barrier (64), offering neuroprotective effects against neuronal damage, particularly due to global and focal cerebral ischemia (65,66). However, the effect of apocynin on brain damage following experimental stroke depends on the dose used (67). Indeed, apocynin, given at a dose of 2.5 mg/kg 30 min before reperfusion, improved neurological function, reduced infarct volume, and reduced the incidence of cerebral hemorrhage. At higher doses of 3.75 and 5 mg/kg, it increased brain hemorrhage (67). Other reports indicate that the therapeutic potential of systemic

**Figure 6.** Effect of apocynin on behavioral alterations induced by social isolation. (A) Locomotor activity in the open field test in control (CTRL) and isolated (ISO) rats not treated and treated with apocynin (APCN; n = 10 per group), HD = horizontal displacements (number of crossings of the black lines), VD = vertical displacements (number of rearing). F(1,36) = 401.83, p < .001; F(3,108) = 489.08, p < .001; F(2,72) = 523.79, p < .001; ***p < .001, NS = nonsignificant (p = .052 CTRL WATER vs. CTRL APCN, p = .589 CTRL APCN vs. ISO APCN). Two-way analysis of variance followed by Tukey post hoc test. (B) Discrimination index in the novel object recognition test in CTRL and ISO rats not treated and treated with APCN (n = 10 per group). (N = times spent in exploration of the novel object in the choice trial; F = times spent in exploration of the familiar object in the choice trial). F(1,36) = 37.92, p < .001; F(1,36) = 56.37, p < .001; F(2,72) = 48.72, p < .001; ***p < .001, n.s. = nonsignificant (p = .565 CTRL WATER vs. CTRL APCN, p = .528 CTRL APCN vs. ISO APCN). Two-way analysis of variance followed by Tukey post hoc test.

Reference:
S. Schiavone et al.
administration of apocynin is in a dose range of 2.5–12 mg/kg in various animal models (68,69).

The mechanistic links between the increase in oxidative stress in the brain and the loss of parvalbumin immunoreactivity is only partially understood. It appears that parvalbumin-positive interneurons do not die but rather change their phenotype and produce less parvalbumin (70). Thus, oxidative stress most likely increases the activity of redox-sensitive transcription factors (71) or indirectly through the modulation of redox-sensitive signaling pathways, such as the NMDA receptor (8).

In conclusion, we demonstrated that oxidative stress, mediated at least in part by NOX2, provides a link between social stress and brain alterations, ultimately leading to abnormal behavior. Thus, NOX enzymes are set to become interesting new players in the mechanisms leading to psychiatric diseases.

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Authors SS and SC are joint first authors and authors LT and K-HK are joint senior authors.

Supplementary material cited in this article is available online.


Supplementary Information

Supplementary Methods

Labeling of the animals

The labeling of animals enrolled in this study was carried out by the animal facility and not by the investigators. Rats were labeled by permanent ink signs in the tail and assigned one alphabetic letter per couple. At the weaning, each pup was labeled with conventional identification ear signs and assigned the alphabetic letter of the parents, followed by a number (e.g. the first pup of the couple A was labeled as rat A1). It was not possible to deduce from the labeling whether an animal was isolated or not.

Enrollment of the animals in the study

Rats were randomly assigned to control or isolation condition by staff of the animal facility. To avoid litter effects, pups of a given litter were equally distributed between control and isolation groups with or without apocynin treatment. The social isolation procedure was performed in the animal facility, not accessible to the investigators. Researchers performing behavioral, histological and biochemical analysis were blind with respect to the rearing and treatment conditions. The blinding of the data was maintained until the analysis was terminated.
Supplementary Table 1. Exploratory time (sec) in the familiarization trial of the novel object recognition test

<table>
<thead>
<tr>
<th>Familiarization Trial</th>
<th>CTRL</th>
<th>EXPLORATORY TIME</th>
<th>ISO</th>
<th>CTRL APCN</th>
<th>ISO APCN</th>
</tr>
</thead>
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<tr>
<td>Object 1</td>
<td>24.7±0.42</td>
<td>27.0±0.68</td>
<td>24.7±0.72</td>
<td>25.8±0.61</td>
<td></td>
</tr>
<tr>
<td>Object 2</td>
<td>25.4±0.43</td>
<td>27.0±0.54</td>
<td>25.1±0.60</td>
<td>24.9±0.43</td>
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<tr>
<td>Total (object 1+object 2)</td>
<td>50.1±0.62</td>
<td>54.3±0.90**</td>
<td>49.8±1.08</td>
<td>50.7±0.82</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.E.M.
CTRL, control rats (n=10); ISO, isolated rats (n=10); CTRL APCN, control rats treated with apocynin (n=10); ISO APCN, isolated rats treated with apocynin (n=10).
Statistical analysis: Two Way ANOVA (post-hoc test: Tukey’s test) on Total (object1+object2):
**p<0.01 vs CTRL, CTRL APCN, ISO APCN
Supplementary Table 2. Exploratory time (sec) in the choice trial of the novel object recognition test

<table>
<thead>
<tr>
<th>Choice trial</th>
<th>EXPLORATORY TIME</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTRL</td>
<td>ISO</td>
<td>CTRL APCN</td>
<td>ISO APCN</td>
</tr>
<tr>
<td>Object F</td>
<td>6.6±0.43</td>
<td>18.3±1.99°°°</td>
<td>6.8±0.53</td>
<td>9.5±1.56</td>
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<tr>
<td>Object N</td>
<td>27.2±3.38***</td>
<td>20.2±2.24</td>
<td>27.1±2.77###</td>
<td>28.1±1.63####</td>
</tr>
<tr>
<td>Total (F+N)</td>
<td>33.8±3.49</td>
<td>38.5±3.69</td>
<td>33.9±3.15</td>
<td>37.6±2.90</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.E.M.
CTRL, control rats (n=10); ISO, isolated rats (n=10);
CTRL APCN, control rats treated with apocynin (n=10); ISO APCN, isolated rats treated with apocynin (n=10); F, familiar; N, novel
Statistical analysis: Three Way ANOVA (post-hoc test: Tukey’s test):
°°°p<0.001 vs CTRL object F, CTRL APCN object F, ISO APCN object F
***p<0.001 vs CTRL object F; vs ISO object N
###p<0.001 vs CTRL APCN object F
####p<0.001 vs ISO APCN object F

Skewness Kurtosis tests were used to check the normalcy of the distribution of the “exploratory time” parameter. Results show that “exploratory time” parameter is normally distributed within each level of each factor.
Supplementary Figures

Supplementary Fig. 1

A  B

C  D

ac  ac

E  F

fmi  fmi
Supplementary Fig. 1. Increase of microglia after social isolation.

(A-B) Representative images of immunohistochemistry for IBA-1 in the PFC of control (A; n=4) and isolated rats (B; n=4).

(C-D) Representative images of immunohistochemistry for IBA-1 in the NACC of control (C; n=4) and isolated rats (D; n=4).

(E-F) Representative images of immunohistochemistry for IBA-1 at the level of the forceps minor of control (E; n=4) and isolated rats (F; n=4).

aca, anterior commisure, anterior part; fmi, forceps minor of the corpus callosum (1).

Scale Bar A-F=275 μm
Supplementary Fig. 2 A-L
Supplementary Fig. 2 M-X
**Supplementary Fig. 2.** Increase of oxidative stress in microglia cells and parvalbumin immunoreactive neurons after social isolation.

(A-F) Representative images of immunofluorescence for 8-OHdG (green fluorescence), IBA-1 (red fluorescence), Dapi (blue fluorescence) in the PFC of control rats (A-C; n=6) and of isolated rats (D-F; n=6).

(G-L) Representative images of immunofluorescence for 8-OHdG (green fluorescence), IBA-1 (red fluorescence), Dapi (blue fluorescence) in the NACC of control rats (G-I; n=6) and isolated rats (J-L; n=6).

(M-R) Representative images of immunofluorescence for 8-OHdG (green fluorescence), parvalbumin (red fluorescence), Dapi (blue fluorescence) in the PFC of control rats (M-O; n=6) and isolated rats (P-R; n=6).

(S-X) Representative images of immunofluorescence for 8-OHdG (green fluorescence), parvalbumin (red fluorescence), Dapi (blue fluorescence) in the NACC of control rats (S-U; n=6) and isolated rats (V-X; n=6).

C, F, I, L, are merged pictures for Dapi staining and IBA-1 and 8OH immunoreactivity.

O, R, U, X are merged pictures for Dapi staining and PV and 8OH immunoreactivity.

Scale bar A-X: 85 μm.
Supplementary Fig. 3
Supplementary Fig. 3. Effect of apocynin treatment on 8-OHdG and c-fos staining in isolated animals.

(A-B) Representative images of immunohistochemistry for 8-OHdG in the PFC of isolated rats not treated (A) and treated with apocynin (B) (n=4 per group).

(C-D) Representative images of immunohistochemistry for 8-OHdG in the NACC of isolated rats not treated (C) and treated with apocynin (D) (n=4 per group).

(E-F) Representative images of immunofluorescence for c-fos (red fluorescence) in the PFC of isolated rats not treated (E) and treated with apocynin (F) (n=4 per group).

(G-H) Representative images of immunofluorescence for c-fos (red fluorescence) in the NACC of isolated rats not treated (G) and treated with apocynin (H) (n=4 per group).

Scale bar A-D: 64 μm, E-H: 126 μm.
aca, anterior commisure, anterior part (1).

3. RESEARCH ARTICLE:

The NADPH Oxidase NOX2 controls glutamate release: a novel mechanism involved in psychosis-like ketamine responses
Subanesthetic doses of NMDA receptor antagonist ketamine induce schizophrenia-like symptoms in humans and behavioral changes in rodents. Subchronic administration of ketamine leads to loss of parvalbumin-positive interneurons through reactive oxygen species (ROS), generated by the NADPH oxidase NOX2. However, ketamine induces very rapid alterations, in both mice and humans. Thus, we have investigated the role of NOX2 in acute responses to subanesthetic doses of ketamine. In wild-type mice, ketamine caused rapid (30 min) behavioral alterations, release of neurotransmitters, and brain oxidative stress, whereas NOX2-deficient mice did not display such alterations. Decreased expression of the subunit 2A of the NMDA receptor after repetitive ketamine exposure was also precluded by NOX2 deficiency. However, neurotransmitter release and behavioral changes in response to amphetamine were not altered in NOX2-deficient mice. Our results suggest that NOX2 is a major source of ROS production in the prefrontal cortex controlling glutamate release and associated behavioral alterations after acute ketamine exposure. Prolonged NOX2-dependent glutamate release may lead to neuroadaptive downregulation of NMDA receptor subunits.

Introduction
Alteration of glutamatergic neurotransmission is thought to be involved in the development of psychosis (Harte et al., 2007). This appears to be attributable to NMDA receptor (NMDAR) hypofunction, which decreases the activity of GABAergic interneurons, leading to an excessive glutamate release (Homayoun and Moghaddam, 2007) and subsequent cortical excitation, as well as schizophrenia-like behavioral and cognitive anomalies (Farber, 2003).

Studies on the effects of the NMDA receptor antagonists, such as ketamine and phencyclidine, support this theory (Olney et al., 1999). Controlled administration of ketamine in healthy volunteers leads to positive, negative, and cognitive symptoms similar to those observed in schizophrenic patients (Krystal et al., 2003). Subanesthetic ketamine administration also induces behavioral alterations in animals, and it is therefore used as a pharmacological animal model of schizophrenia (Bubeniková-Valesová et al., 2008; Gunduz-Bruce, 2009). Although psychotic symptoms are impossible to evaluate and replicate in rodents, alteration of motor behavior can be used as a read-out for the response to NMDA receptor antagonists (Krystal et al., 2003; Imre et al., 2006; Gunduz-Bruce, 2009). The effect of ketamine is mainly attributable to the activation of the prefrontal cortex and the limbic structures (Jentsch and Roth, 1999; Imre et al., 2006), in which increased levels of glutamate and dopamine have been detected after acute ketamine injections (Gunduz-Bruce, 2009). In addition, chronic exposure to low doses of ketamine leads to phenotypic changes in neurons, such as the loss of parvalbumin in GABAergic interneurons (Cochran et al., 2003; Kinney et al., 2006), also observed in schizophrenic patients (Lewis et al., 2005). Corroborating these results, genetic ablation of the NMDA receptor also confers schizophrenia-like phenotypes in mice (Gainetdinov et al., 2001; Belforte et al., 2010).

Emerging evidence indicates a contribution of reactive oxygen species (ROS) to the development of schizophrenia. Altered antioxidant defense and signs of oxidative insults are observed in patient samples (Yao et al., 2001). In this context, it has been shown that the activation of the ROS-producing NADPH oxidase NOX2 enzyme contributes to dysfunction of GABAergic interneurons after subchronic ketamine exposure in mice (Behrens et al., 2007, 2008). We have also recently demonstrated the involvement of NOX2 in behavioral and biochemical alterations caused by social isolation in rats (Schiavone et al., 2009). These two different animal models induce chronic neural stress and, in both cases, NOX2 upregulation appears to be responsible for the prolonged oxidative insult on GABAergic neurons. However, it has
been reported that acute ketamine administration causes immediate formation of ROS (Zuo et al., 2007) and oxidative damage (de Oliveira et al., 2009). The mechanism of this rapid ROS generation is not known. Possible sources of ROS include NOX enzymes, which are constitutively expressed in the brain and are major generators of ROS in pathological conditions (Sorce and Krause, 2009). The aim of this study was to understand how NOX2-deficient ROS production participates in the rapid modifications induced by acute ketamine administration. Our data show that the immediate behavioral and neurochemical abnormalities induced by ketamine are prevented in NOX2-deficient knock-out (KO) mice and that NOX2 is involved in neurotransmitter release.

Materials and Methods

Mice. NOX2-deficient mice in the C57BL/6 background were purchased from The Jackson Laboratory. Mice were given access ad libitum to food and water in a quiet room at 25°C with a 12 h light/dark cycle. Mice were housed in groups of three to four. All experiments were performed using wild-type (WT) and KO NOX2 mice male littermates of 8–10 weeks of age. All experiments were performed according to institutional guidelines (Italian Legislative Decree 116, Official Journal of the Italian Republic, supplement 40, 18 February 1992; Ministerial Memorandum No. 8, Official Journal of the Italian Republic, 14 July 1994; EEC Council Directive 86/609, Official Journal Law 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996), and approved by the ethical committee and the veterinary office of the University of Foggia.

Behavioral analysis: open-field test. The apparatus consisted of a circular arena, made of dark plastic located in a silent experimental room illuminated. Habituation trial was performed over 5 d before the test in the same experimental conditions. Animals were acclimated to the test room for 1 h before each test. The day of the test, mice were injected intraperitoneally with ketamine (30 mg/kg; Sigma-Aldrich), amphetamine (1 mg/kg; Sigma-Aldrich), or saline solution, as control. Drug doses were chosen according to previous reports (for ketamine, Iriúne et al., 1991, 1998; Behrens et al., 2007; and for amphetamine, Sansone, 1980; Spielevero et al., 2001; Coitinho et al., 2002). Behavioral test was performed 30 min and 4 h after injection. Motor activity was measured by placing the mouse into the center of the arena for a 20 min session. The scoring was performed using a specific software (Observer 3.0; Noldus Information Technology). To assess general locomotor activity, the following behavioral parameters (expressed as frequency in 20 min) were scored: number of square limit crossings with both forepaws, rearing (standing with the body inclined vertically, forequarters raised), total grooming activity consisting of face grooming (strokes along the snout), head washing (semicircular movements over the top of the head and behind the ears), and body grooming (body fur licking), and total sniffing (deep smelling activity both on the floor and on the walls of the arena). Behavioral test was performed by an investigator who was blind to the animal experimental group.

Immunohistochemistry. Mice were perfused with a solution of 4% paraformaldehyde in PBS, pH 7.35, and brains were removed, paraffin embedded, and cut in 10-μm-thick sections.

Immunohistochemistry was performed as described previously (Schiafone et al., 2009). Briefly, after deparaffinization and heat-induced epitope retrieval, brain sections were incubated overnight at 4°C with one or two (for double staining) primary antibodies at the following concentrations: 8-hydroxy-2′-deoxyguanosine (8-OHdG), 1:10 (JalCA); NMDAR subunit 2A (NMDAR-2A), 1:400 (Abcam); and NMDAR-2B, 1:200 (Abcam). For 3,3′-diaminobenzidine (DAB) immunohistochemistry, sections were then incubated first for 1 h at room temperature with a biotinylated secondary antibody (Vector Laboratories) and then with the ABC complex solution (Vector Laboratories). Tissue-bound peroxidase was visualized using DAB (Sigma-Aldrich) and H2O2 (Sigma-Aldrich). For immunofluorescence detection, sections were incubated with specific fluorescent-labeled secondary antibody (1:1000, Alexa Fluor 488 or 555; Invitrogen). 4′,6′-Diamidino-2-phenylindole (DAPI) counterstaining was performed to visualize cell nuclei. Imaging was performed on an LSM 510 Meta confocal laser scanner mounted on an Axio Imager Z1 microscope (Carl Zeiss). Negative controls consisting of tissue incubated without primary antibodies were performed for each experiment (data not shown).

Real-time quantitative PCR. Total RNA was isolated using RNeasy mini kit (Qiagen) according to the instructions of the manufacturer. Residual genomic DNA was removed using RNase-Free DNase set (Qiagen). Total RNA (1 μg) was reverse transcribed using the superscript II kit according to the instructions of the manufacturer (Invitrogen). Real-time quantitative PCR reactions were performed using Power SYBR Green PCR master mix (Applied Biosystems) and a Chromo 4TM Real-Time system (Bio-Rad). Quantification was performed at a threshold detection line (Ct value). The Ct value of each target genes (c-fos, parvalbumin) was normalized with relative levels of Rp59 (ribosomal protein S9) and Tbp (TATA-box binding protein) mRNAs used as housekeeping genes. Triplicates were performed for each condition. The list of the primers used is given in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

Semi-quantitative end-point PCR. Extraction of RNA and reverse transcription were performed as described above. Semi-quantitative end-point PCR was performed by determining the suitable number of PCR cycles giving linear cDNA amplification of each gene of interest using TaqDNA polymerase (Qiagen). Amplification of the housekeeping gene encoding the L32 ribosomal protein was used as control. The list of the primers used is given in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

Microdialysis. Mice were anesthetized intraperitoneally with 3.6 ml/kg Equithesin (composition: 1.2 g of sodium pentobarbital, 5.3 g of chloral hydrate, 2.7 g of MgSO4. 49.5 ml of propylene glycol, 12.5 ml of ethanol, and 58 ml of distilled water) and secured in a stereotactic frame (David Kopf Instruments). The skin was shaved, disinfected, and cut with a sterile scalpel to expose the skull. A vertical dialysis probe was positioned in the prefrontal cortex of mice (Castane et al., 2008): from bregma, anteroposterior, ±2.2 mm and mediolateral, ±0.2 mm; and from the skull surface, the extremity of the probe was pushed in dorsoventral, −3.4 mm. On the day of the experiment (20–24 h after implantation of the probes), microdialysis was performed in freely moving mice as described previously (Castane et al., 2008) by perfusing the fibers with an artificial CSF containing the following (in mM): 145 NaCl, 3 KCl, 1.26 CaCl2, 1 MgCl2, and 1.4 Na2HPO4 in distilled water; the solution was buffered at pH 7.3 with 0.6 mM NaH2PO4 and filtered (0.45 μm). The fibers were perfused at a constant flow rate of 1 μl/min using a CMA/100 microinjection pump (CMA Microdialysis). In all experiments, the microdialysis membrane was allowed to stabilize for 2 h at the flow rate of 1 μl/min without collecting samples. At the end of the stabilization period, samples were collected at a flow rate of 1 μl/min. Three baseline samples were collected to evaluate baseline release of neurotransmitters. After this time, ketamine (30 mg/kg, i.p.), amphetamine (1 mg/kg, i.p.), or vehicle (saline) injections were performed, and additional samples were collected. WT and KO NOX2 mice were treated and analyzed at the same time, but data in figures are presented as separate panels for better clarity. The position of the microdialysis probe was verified by histological procedures at the end of each experiment. Only mice in which probe tracks were exactly located in the target area were considered in Results.

Quantification of glutamate in the dialysate. Glutamate concentrations were determined by HPLC using ODS-3 column (150 × 4.6 mm, 3 μm; INERTSIL) with fluorescence detection after derivatization with o-phthalaldehyde/mercaptopropionic acid (emission length, 4.60 nm; excitation length, 3.40 nm). The mobile phase gradient consisted of 50 mM sodium acetate buffer, pH 6.95, with methanol increasing linearly from 2 to 30% (v/v) over 40 min (Morrone et al., 2007). The flow rate was 0.5 ml/min. Results were analyzed by Borwin software (version 1.50; Jasco). Because no difference in extracellular basal levels of glutamate between experimental groups was detected (for original values and statistics, see supplemental Table 2, available at www.jneurosci.org as supplemental material), results were expressed as percentage of baseline.

Quantification of dopamine in the dialysate. Dopamine concentrations were determined by HPLC using LC-18 DB column (150 × 4.6 mm, 5 μm; SUPELCOSIL) with ESA Coulometric detection (ESA) in oxidation/
were analyzed by Chromeleon software (version 6.60; Dionex). Because no ratio of 2) (Bassareo and Di Chiara, 1997; Trabace et al., 2007). Results detection limit was buffered at pH 5.8 with 1N NaOH. The flow rate was 1 ml/min, and ylamine, 15% acetonitrile, 13% methanol, solved in distilled water and as statistically significant. Results are expressed as means

\[ p = 0.001; \]

\[ p < 0.001; \]

\[ p < 0.01; \]

\[ p < 0.05. \]

**Results**

**NOX2 deficiency prevents ketamine-induced behavioral abnormalities**

An open-field test was performed 30 min after ketamine injection. Locomotor activity was evaluated as the frequency of crossings, rearings, grooming, and sniffings. In WT mice, all these parameters were markedly increased compared with saline control mice. In contrast, ketamine did not elicit similar responses in KO NOX2 mice because their locomotor behavior remained un-

**Stress-related markers after ketamine injection**

Ketamine administration causes an increase in ROS production (Zuo et al., 2007) and oxidative damage (de Oliveira et al., 2009) in the brain. However, the source of the ROS generation is not known. Indirect markers of oxidative stress were measured 30 min after ketamine injection in WT and KO NOX2 mice. The staining for the DNA oxidation marker 8-OHdG was increased in the prefrontal cortex of WT mice and especially localized in cell nuclei. In contrast, no increase of 8-OHdG was detected in KO NOX2 mice (Fig. 2A–D). Consistent with the role of NOX2 as source of ROS in this condition, NOX2 and all the subunits required for its activity were detected in the prefrontal cortex (supplemental Fig. 2A, B, available at www.jneurosci.org as supplemental material). It is also known that ketamine injections leads to the upregulation of the transcription factor subunit c-fos

**Figure 1.** Behavioral alterations induced by ketamine exposure are prevented in NOX2-deficient mice. Thirty minutes after injection with ketamine (30 mg/kg, i.p.) or saline, mice were placed in the arena for open-field test. A–D, Bar graphs represent the frequency of grooming (A), rearing (B), crossing (C), and sniffing (D) recorded during the 20 min of the test in WT and KO NOX2 mice. For grooming: Treatment (tr) \( \times \) Genotype (gen) \( \times \) Treatment \( \times \) Genotype analysis of variance (ANOVA) followed by Tukey’s post hoc test (\( p < 0.05 \)).

**Figure 2.** Stress markers are increased after ketamine injections in wild-type mice, and not in NOX2-deficient mice. A–D, Representative images of immunohistochemistry staining for 8-OHdG in the prefrontal cortex of WT (A, B) or KO NOX2 (C, D) mice treated with saline (A, C) or ketamine (B, D), \( n = 5 \) per condition. Scale bar, 65 \( \mu \)m. E, Real-time PCR quantification of the immediate early gene c-fos mRNA in prefrontal cortex after ketamine or saline injection in WT and KO NOX2 mice. RT \( \times \) Gen \( \times \) Treatment analysis of variance (ANOVA) followed by Tukey’s post hoc test (\( p < 0.05 \)).

**Figures:** A–D, available at www.jneurosci.org as supplemental material. Results are expressed as means ± SEM.

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**Supplemental material:** available at www.jneurosci.org as supplemental material.
Ketamine-induced release of extracellular glutamate and dopamine depends on NOX2

Alterations of neurotransmitter levels in the cortex induced by ketamine are thought to determine psychotic symptoms (Gunduz-Bruce, 2009). To understand the cause of the different behavioral response between WT and KO NOX2 mice, we have performed microdialysis in freely moving animals to measure the extracellular concentration of neurotransmitters in mouse brain. Basal levels of glutamate were comparable in WT and KO NOX2 mice. After ketamine injection, an immediate increase in glutamate level was detected in the prefrontal cortex of WT mice. The elevated concentration was observed after 30 min and persisted during the subsequent 4 h from the injection (Fig. 3A). In contrast, ketamine did not elicit the same response in KO NOX2 mice because glutamate concentration remained at basal levels (Fig. 3B). Glutamatergic and dopaminergic systems are closely interconnected (Sesack et al., 2007). We have therefore quantified c-fos mRNA expression by real-time PCR. In WT mice, c-fos expression levels were increased in the prefrontal cortex after ketamine injection. In contrast, no change in c-fos expression was seen in mice lacking NOX2 (Fig. 2E). Under the same conditions, we have also measured the level of parvalbumin mRNA. Whereas subchronic exposure to ketamine induces a decrease in parvalbumin (Behrens et al., 2009), we did not detect a reduction in parvalbumin mRNA after acute ketamine exposure in either control nor KO NOX2 mice (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Thus, c-fos expression is an early NOX2-dependent event in response to ketamine, whereas the decrease in parvalbumin appears to occur only at later time points (Behrens et al., 2007).

Glutamate and dopamine level elevation is prevented in NOX2-deficient mice.

Figure 3. Glutamate and dopamine level elevation is prevented in NOX2-deficient mice. A–D, Time-dependent effect of ketamine or saline injection on extracellular glutamate (GLU; A, B) and dopamine (DA; C, D) levels was determined by microdialysis in the prefrontal cortex of WT (A, C) and KO NOX2 (B, D) mice. Data are expressed as the percentage of baseline (as described in Materials and Methods). E, F, Concentration of glutamate (E) and dopamine (F) in WT and KO NOX2 mice at basal level or 30 min after saline or ketamine injection. ***p < 0.001 using two-way ANOVA for repeated measures followed by Tukey’s post hoc test (n = 6 WT saline and ketamine; n = 4 KO NOX2 saline and ketamine). For glutamate analysis: two-way ANOVA for repeated measures in KO NOX2 saline versus KO NOX2 ketamine: Fr(1,44) = 2.259, p = 0.184; F(time) = 1.882, p = 0.058; Fr × gen(1,44) = 0.568, p = 0.847; in WT ketamine versus KO NOX2 ketamine: F(gen) = 345.017, p < 0.001; F(time) = 327.118, p < 0.001; in WT ketamine versus KO NOX2 saline: F(time) = 7557.223, p < 0.001; F(gen) = 358.748, p < 0.001; F(time × gen) = 329.535, p < 0.001; in WT saline versus KO NOX2 ketamine: F(time) = 15.944, p = 0.004; F(time × gen) = 2.118, p = 0.027; F(time × f) = 0.551, p = 0.863; however, Tukey’s post hoc test did not reveal any significant difference for treatment and time; in WT saline versus KO NOX2 saline: F(gen) = 0.00160, p = 0.969; F(time × gen) = 2.011, p = 0.036; F(time × gen) = 0.443, p = 0.932; however, Tukey’s post hoc test did not reveal any significant difference for genotype and time; in WT ketamine versus WT saline: F(time) = 16.702.806, p < 0.001; F(gen) = 529.138, p < 0.001; F(time × gen) = 505.459, p < 0.001. For dopamine analysis: two-way ANOVA for repeated measures in KO NOX2 saline versus KO NOX2 ketamine: F(gen) = 0.000525, p = 0.945; F(time) = 0.633, p = 0.749; F(time × gen) = 0.440, p = 0.932; in WT ketamine versus KO NOX2 saline: F(time) = 360.147, p < 0.001; F(gen) = 16.156, p < 0.001; F(time × gen) = 14.890, p < 0.001; in WT saline versus KO NOX2 ketamine: F(time) = 0.00714, p = 0.935; F(gen) = 0.480, p = 0.911; F(time × gen) = 0.37, p = 0.965; in WT saline versus KO NOX2 saline: F(time) = 0.00000392, p = 0.998; F(gen) = 0.31, p = 0.982; F(time × gen) = 0.234, p = 0.994; in WT ketamine versus WT saline: F(time) = 20.573, p < 0.001; F(gen) = 18.919, p < 0.001; in WT ketamine versus KO NOX2 ketamine: F(time) = 14.634, p < 0.001; F(gen) = 15.363, p < 0.001; F(time × gen) = 15.068, p < 0.001.
Amphetamine induces similar response in WT and KO NOX2 mice

To verify whether the lack of neurotransmitter increase after ketamine injection was attributable to a general defect in KO NOX2 mice, the response to amphetamine, another psychostimulant drug, was evaluated. In fact, amphetamine is known to induce a rapid efflux of dopamine from the ventral tegmental area toward the prefrontal cortex, in which subsequent glutamate is similarly released (Sesack et al., 2003; Sulzer et al., 2005). As illustrated in Figure 4, A and B, amphetamine injection caused a large increase in dopamine levels in both WT and KO NOX2 mice. Consequently, glutamate concentration was also elevated (Fig. 4 C,D). Thus, NOX2-dependent alteration of glutamatergic and dopaminergic release is a specific response to ketamine. In addition, we have evaluated the behavioral alterations induced by amphetamine. In agreement with the microdialysis data, WT and KO NOX2 mice displayed a similar increase of locomotor activity after amphetamine injection (Fig. 5). Hence, NOX2 deficiency does not prevent behavioral changes in response to amphetamine.

Downregulation of the NMDA receptor subunit 2A after repeated ketamine injections is prevented in KO NOX2 mice

Excessive glutamate increase after repetitive administration of ketamine can lead to the downregulation of postsynaptic receptors. Therefore, we have analyzed the presence of NMDAR-2A and NMDAR-2B in the prefrontal cortex and in the posterior cingulate cortex. Both of these brain regions are subject to alterations in response to prolonged ketamine exposure (Ellison, 1995; Olney et al., 1999; Tsai and Coyle, 2002). As shown in Figure 6, WT mice displayed reduced expression of the NMDAR-2A after repeated ketamine injections (Fig. 6, A vs B and E vs F). In contrast, KO NOX2 mice displayed similar immunostaining for NMDAR-2A after ketamine or saline injections (Fig. 6, C vs D and G vs H). The
difference for time; in WT amphetamine versus KO NOX2 saline: $F_{11,55} = 42.943, p < 0.001$; $F_{11,55} = 7.117, p < 0.001$; in WT saline versus KO NOX2 amphetamine: $F_{11,55} = 108.426, p < 0.001$; $F_{11,55} = 9.661, p < 0.001$; in WT saline versus KO NOX2 saline: $F_{11,55} = 10.743, p < 0.001$; in WT saline versus KO NOX2 saline: $F_{11,55} = 0.0686, p = 0.802$; $F_{11,55} = 0.814, p = 0.999$; $F_{11,55} = 0.362, p = 0.966$; in WT amphetamine versus WT saline: $F_{11,55} = 35.877, p < 0.001$; $F_{11,55} = 6.078, p < 0.001$; $F_{11,55} = 6.594, p < 0.001$.

Figure 4. Neurotransmitter level elevation is similar in wild-type and NOX2-deficient mice after amphetamine exposure. A–D, Time-dependent effect of amphetamine or saline injection on extracellular dopamine (DA; A, B) and glutamate (GLU; C, D) levels was determined by microdialysis in the prefrontal cortex of WT (A, C) and KO NOX2 (B, D) mice. Data are expressed as the percentage of baseline (as described in Materials and Methods). E, F, Concentration of dopamine (E) and glutamate (F) in WT and KO NOX2 mice at basal level or 30 min after saline or amphetamine injection. *$p < 0.001$ using two-way ANOVA for repeated measures followed by Tukey’s post hoc test ($n = 4$ WT saline; $n = 4$ WT amphetamine; $n = 4$ KO NOX2 saline; $n = 4$ KO NOX2 amphetamine). For dopamine analysis: two-way ANOVA for repeated measures in KO NOX2 saline versus KO NOX2 amphetamine: $F_{11,66} = 179.126, p < 0.001$; $F_{11,66} = 62.712, p < 0.001$; $F_{11,66} = 63.847, p < 0.001$; in WT amphetamine versus KO NOX2 amphetamine: $F_{11,66} = 0.282, p = 0.615$; $F_{11,66} = 62.241, p < 0.001$; $F_{11,66} = 0.413, p = 0.945$; however, Tukey’s post hoc test did not reveal any difference for time; in WT amphetamine versus KO NOX2 saline: $F_{11,66} = 28.066, p = 0.002$; $F_{11,66} = 18.176, p < 0.001$; $F_{11,66} = 16.651, p < 0.001$; in WT saline versus KO NOX2 amphetamine: $F_{11,66} = 198.908, p < 0.001$; $F_{11,66} = 52.993, p < 0.001$; $F_{11,66} = 63.514, p < 0.001$; in WT saline versus KO NOX2 saline: $F_{11,66} = 0.219, p = 0.642$; $F_{11,66} = 1.854, p = 0.062$; $F_{11,66} = 0.753, p = 0.684$; in WT amphetamine versus WT saline: $F_{11,66} = 30.049, p = 0.002$; $F_{11,66} = 16.115, p < 0.001$; $F_{11,66} = 19.754, p < 0.001$. For glutamate analysis: two-way ANOVA for repeated measures in KO NOX2 saline versus KO NOX2 amphetamine: $F_{11,55} = 423.188, p < 0.001$; $F_{11,55} = 14.986, p \leq 0.001$; $F_{11,55} = 14.751, p \leq 0.001$; in WT amphetamine versus KO NOX2 amphetamine: $F_{11,55} = 0.490, p = 0.515$; $F_{11,55} = 20.271, p < 0.001$; $F_{11,55} = 0.246, p < 0.993$; however, Tukey’s post hoc test did not reveal any
The presence of NOX2 in the brain has been widely documented. In particular, NOX2 is expressed in microglia, astrocytes, and neurons (Sorce and Krause, 2009). In neutrophils, NOX2 activity is tightly regulated and, during activation, requires the phosphorylation of specific cytosolic subunits and their translocation to the membrane to generate ROS (Bedard and Krause, 2007). The presence in the brain of all the cytosolic subunits required for NOX2 activation suggests that formation of the enzymatic complex can rapidly occur after ketamine exposure. Signs of oxidative stress were observed only in the brain of wild-type mice. Because behavioral alterations do not occur in NOX2-deficient mice, it appears that NOX2 can rapidly generate ROS in response to ketamine. Basically, the ketamine-induced NOX2 activation could be either a direct consequence of NMDA receptor inhibition or attributable to another pathway. The fact that two NMDA receptor antagonists, ketamine and dizocilpine [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate], both induce cerebral ROS generation during injection in rats (de Oliveira et al., 2009) and mice (Zuo et al., 2007) support the former possibility. However, given the lack of cellular studies specifically addressing this issue, it is, at this point, difficult to exclude that ketamine activates NOX2 through other mechanisms.

There are conflicting data in the literature concerning the duration of ketamine-induced behavioral alterations. Short-term (<2 h) behavioral changes in response to ketamine have been described previously (Hayase et al., 2006), whereas other observations suggest a markedly longer persistence (Uchihashi et al., 1994; Irifune et al., 1998). In our model, 4 h after ketamine injection, wild-type mice still displayed a marked enhancement of crossing, grooming, rearing, and sniffing. This is consistent with the prolonged elevations of neurotransmitters observed in our study.

ROS are important signaling molecules involved in physiological mechanisms in the brain, such as synaptic plasticity and memory (Kishida and Klann, 2007). In particular, ROS are able to modulate neurotransmission in cerebral circuitry. For example, the glutamate and GABA-controlled release of dopamine is dependent on hydrogen peroxide produced by mitochondria in the striatum (Avshalumov et al., 2003; Bao et al., 2009). Therefore, it is conceivable that one function of NOX2 in the brain is to produce ROS for the control of specific synaptic neurotransmission. Basal levels of glutamate and dopamine are similar in wild-type and NOX2-deficient mice, suggesting that absence of NOX2 does not impair normal synaptic activity. However, the fact that ketamine response is prevented in NOX2-deficient mice argues in favor of a NOX2-dependent ROS production that mediates neurochemical and behavioral alterations. This NOX2-dependent mechanism is specific for ketamine because the response to amphetamine in NOX2-deficient mice was similar to wild-type mice. Corroborating our results, Kishida et al. (2006) found an intact basal synaptic activity after pharmacological inhibition or genetic ablation of NOX2 but impairment in NMDA receptor-dependent long-term potentiation and memory formation, which require an increased release of glutamate (Kishida et al., 2006).

In addition to an early effect on the development of psychosis, it is tempting to speculate that NOX2-dependent elevation of glutamate levels induced by ketamine determines long-term alteration on neuronal connectivity. Indeed, the inhibition of GABAergic neurons by NMDA receptor antagonism eventually leads to their hyperstimulation because of the disinhibited glutamatergic inputs. This excessive stimulation of postsynaptic neurons is known to induce neurochemical and morphological changes, similar to those observed in schizophrenic patients (Farber, 2003). In this context, our data show that a repeated

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**Discussion**

In this study, the involvement of NOX2 on acute behavioral and neurochemical effects elicited by ketamine administration was analyzed. Our results show that NOX2-deficient mice are completely protected from the behavioral alterations induced by ketamine. In addition, they do not display signs of oxidative stress in the brain and increased extracellular levels of glutamate and dopamine after ketamine administration. Thus, our data suggest a critical role of NOX2 activation in the development of psychotic symptoms after ketamine administration, through a direct effect on glutamate and dopamine elevation.

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**Figure 5.** Behavioral alterations are similar in wild-type and NOX2-deficient mice after amphetamine exposure. Thirty minutes after injection with amphetamine (1 mg/kg, i.p.) or saline, mice were placed in the arena for open-field test. A–D, Bar graphs represent the frequency of groomings (A), rearings (B), crossings (C), and sniffings (D) recorded during the 20 min of the test in WT and KO NOX2 mice. For groomings: F\(_{r(3),11}\) = 2.299, p = 0.147; F\(_{r(3),11}\) = 415.537, p < 0.001; F\(_{r(3),11}\) = 1.123, p = 0.312. ***p < 0.001 WT saline versus WT amphetamine; WT amphetamine and KO NOX2 saline versus KO NOX2 amphetamine; NS: WT saline versus KO NOX2 saline, p = 0.837; WT amphetamine versus KO NOX2 amphetamine, p = 1.000. For rearings: F\(_{r(3),11}\) = 0.363, p = 0.703; F\(_{r(3),11}\) = 21.464, p < 0.001; F\(_{r(3),11}\) = 0.0546, p = 0.819; **p < 0.01 WT saline versus WT amphetamine; *p < 0.05 KO NOX2 saline versus KO NOX2 amphetamine; NS: WT saline versus KO NOX2 saline, p = 0.485; WT amphetamine versus KO NOX2 amphetamine, p = 0.862. For crossings: F\(_{c(3),11}\) = 0.00361, p = 0.996; F\(_{c(3),11}\) = 13.164, p = 0.001; F\(_{c(3),11}\) = 0.114, p < 0.741; ***p < 0.001 WT saline versus WT amphetamine; **p < 0.01 WT saline versus WT amphetamine; *p < 0.05 KO NOX2 saline versus KO NOX2 amphetamine; NS: WT saline versus KO NOX2 saline, p = 0.796; WT amphetamine versus KO NOX2 amphetamine, p = 0.854. For sniffings: F\(_{n(3),11}\) = 1.028, p = 0.327; F\(_{n(3),11}\) = 20.887, p < 0.001; F\(_{n(3),11}\) = 0.785, p = 0.390; NS: WT saline versus KO NOX2 saline, p = 0.927; KO NOX2 saline versus KO NOX2 ketamine, p = 0.211. ***p < 0.001; **p < 0.01; *p < 0.05 using two-way ANOVA followed by Tukey’s post hoc test (n = 5 WT saline; n = 5 WT amphetamine; n = 4 KO NOX2 saline; n = 5 KO NOX2 amphetamine).
Figure 6. Expression of the NMDAR-2A and NMDAR-2B in wild-type and NOX2-deficient mice after repeated ketamine injections. Mice were treated with ketamine two times within an interval of 24 h and killed 18 h after the last injection. A–D, Representative images of immunohistochemistry for NMDAR-2A protein in the prefrontal cortex. NMDAR-2A staining in wild-type (A, B) and NOX2-deficient (C, D) mice treated with saline (A, C) or ketamine (B, D). n = 4 per group. E–H, Representative images of immunohistochemistry for NMDAR-2A protein in the posterior cingulate cortex. NMDAR-2A staining in wild-type (E, F) and NOX2-deficient (G, H) mice treated with saline (E, G) or ketamine (F, H). n = 4 per group. I–L, Representative images of immunohistochemistry for NMDAR-2B protein in the prefrontal cortex. NMDAR-2B staining in wild-type (I, J) and NOX2-deficient (K, L) mice treated with saline (I, K) or ketamine (J, L). n = 4 per group. M–P, Representative images of immunohistochemistry for NMDAR-2B protein in the posterior cingulate cortex. NMDAR-2B staining in wild-type (M, N) and NOX2-deficient (O, P) mice treated with saline (M, O) or ketamine (N, P). n = 4 per group. NMDAR-2A/DAPI, NMDAR-2B/DAPI, merged images for NMDAR-2A or NMDAR-2B immunoreactivity and DAPI staining. Scale bar, 40 μm.
exposure to ketamine leads to a decrease in NMDA receptor 2A expression, which is prevented in NOX2-deficient mice. The downregulation of the NMDA receptor subunit 2A, but not 2B, is not specific for interneurons but likely occurs also in other neuronal subtypes. The downregulation of NMDA receptor subunit 2A is possibly attributable to sustained excessive glutamate concentration seen in wild-type mice. In adults, NMDA receptor 2A and 2B subunits are mainly expressed in cortex and hippocampus, in which they regulate numerous neuronal functions and, in particular, synaptic plasticity (Yashiro and Philpot, 2008). NMDA receptor subunit 2A is mainly localized in synaptic sites, whereas the 2B subunit is more expressed in extrasynaptic sites, and alterations of the NMDAR-2A/2B ratio influence neuronal activity and modify cortical functions (Yashiro and Philpot, 2008). The NMDA receptor subunit 2A is prone to adaptive responses in different models of glutamate and NMDA over-stimulation (Wu et al., 2005; Gascón et al., 2008). Also, the NMDAR-2A subunit has relevant features for a role in ketamine-induced neurochemical alterations: (1) decrease of parvalbumin expression after prolonged ketamine exposure is specifically attributable to blockade of the NMDAR-2A subunit (Kinney et al., 2006), (2) the ratio of NMDAR-2A/NMDAR-2B subunits in GABAergic interneurons, specifically in parvalbumin-positive interneurons, is higher than in neighboring pyramidal neurons (Kinney et al., 2006; Xi et al., 2009), and (3) the NMDA receptors containing the 2A subunit contribute to the maintenance of parvalbumin and GAD67 phenotype in interneurons (Kinney et al., 2006). Thus, the observed adaptive reduction of NMDAR-2A could explain the loss of parvalbumin, observed after subchronic ketamine exposure (Behrens et al., 2008) or persistent social isolation rearing (Schiavone et al., 2009). In addition, lower density of NMDA receptor subunit 2A, but not 2B has been described in postmortem brain samples of patients with schizophrenia (Mechri et al., 2001; Beneyto and Meadow-Woodruff, 2008).

The fact that NOX2 inhibition prevents both immediate and adaptive behavioral and neurochemical effects of ketamine raises the question whether a pharmacological inhibition of NOX2 represents a valuable therapeutic approach for schizophrenia. Indeed, decreasing the effect of NMDA receptor antagonists has been proposed to be a key approach for identifying such novel therapies (Krystal et al., 2003). Patients with genetic alteration of NOX2 present decreased levels of specific oxidative stress markers, such as isoprostanes (Violi et al., 2006, 2009). The same markers of oxidative stress are significantly increased in physiological fluids of schizophrenic patients (Dietrich-Muszalska and Olas, 2009). Even a single episode of psychosis induces such an increase (Mahadik et al., 1998; Khan et al., 2002; Zhang et al., 2009), indicating that oxidative damage occurs very early in the course of schizophrenia. The present study suggests that NOX2 is the source of this early generation of ROS, which is associated with the onset and the progression of schizophrenia. Therefore, targeting NOX2 in the CNS could be useful to prevent the development of the disease. A possible side effect of prolonged NOX2 inhibition would be reducing the ability of the neutrophils to kill some microorganisms and the development of local inflammatory reactions (Schäppi et al., 2008). Indeed, the complete absence of NOX2 function results in chronic granulomatous disease (CGD), a hereditary disease characterized by the development of granulomas and by a susceptibility to certain fungal and bacterial infections (Schäppi et al., 2008). However, CGD patients in which only 5–10% of neutrophils generate ROS do not develop obvious symptoms (Kume and Dinauer, 2000). Thus, it appears that there is a good therapeutic window for such a putative therapy. However, although NOX enzymes are increasingly recognized as therapeutic targets, no specific NOX2 inhibitor is available so far for use in humans (Jaquet et al., 2009).

Although ketamine represents mostly a model of psychosis, there is also an interesting connection with depression. A single subanesthetic dose of ketamine has rapid and robust anti-depressant effects in depressed patients, which might be attributable to ketamine-induced glutamate release (Hashimoto, 2009; Skolnick, 2009). Similar effects of ketamine have been observed in mouse models of depression (Maeng and Zarate, 2007; Maeng et al., 2008; Mathew et al., 2008). The role of NOX2 activation in the antidepressant effect of ketamine remains to be solved. Future research should also aim at understanding whether NOX2 controls glutamate release in physiological events, e.g., learning, and other pathological processes, such as neurodegenerative disorders (Sorce and Krause, 2009).

References


**Supplementary Material**

Supplementary Table 1

Oligonucleotide primers and conditions used for real-time PCR (qPCR) and semi-quantitative end-point RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Product size</th>
<th>Tm</th>
<th># cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>c-fos</em> (q-PCR)</td>
<td>F: GGGACAGCCCTTCCACTACC R: GATCTGGCAAAATGCTGT</td>
<td>74</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Parvalbumin</em> (q-PCR)</td>
<td>F: GGACATCAAGACCGGATAGGA R: CACCACCTGGAAGAATTTTG</td>
<td>74</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Tbp</em> (qPCR)</td>
<td>F: TTGCACCTAAAGACCTGTGCCTTC R: TTCTCTAGATGCAGCAGCAA</td>
<td>78</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Rps9</em> (qPCR)</td>
<td>F: GACCAGAAGCTAAAGTGTTGGA R: TCTTGCCAGGTTAAGTTGA</td>
<td>81</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>NOX2</em></td>
<td>F: TCACTACTCAAGGTGATGTATGG R: CAGATATCTAAATTATGTGCTCTCAA</td>
<td>652</td>
<td>58</td>
<td>35</td>
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<tr>
<td><em>p47phox</em></td>
<td>F: CGGAAGAGTTCCGGGAACAG R: ATGACCTCATGGCTGGCTCACC</td>
<td>268</td>
<td>60</td>
<td>35</td>
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<tr>
<td><em>p67phox</em></td>
<td>F: CCTCAACATTGGCTGTTGA R: ACCTCAAGCAAGCACTTT</td>
<td>248</td>
<td>60</td>
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<tr>
<td><em>p40phox</em></td>
<td>F: CAAACAAAGGGAGGTCCA R: GTTTGGCGCCATGTAGACT</td>
<td>160</td>
<td>60</td>
<td>35</td>
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<tr>
<td><em>p22phox</em></td>
<td>F: AAAGAAGAAAAGGGGTCCA R: TAGACTCTTGGAGTCTCCAC</td>
<td>238</td>
<td>60</td>
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<td><em>L32</em></td>
<td>F: GTGAAGCCCAAGTCTGCTCA R: TTGTTGACTCTGATGGCCAG</td>
<td>349</td>
<td>58</td>
<td>30</td>
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</table>
Supplementary Table 2

a) Mean of GLU basal levels (µM) ± SEM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SALINE</td>
<td>0.62 ± 0.01</td>
</tr>
<tr>
<td>WT KETAMINE</td>
<td>0.62 ± 0.004</td>
</tr>
<tr>
<td>KO NOX2 SALINE</td>
<td>0.63 ± 0.01</td>
</tr>
<tr>
<td>KO NOX2 KETAMINE</td>
<td>0.61 ± 0.01</td>
</tr>
</tbody>
</table>

Two way ANOVA for repeated measures:

WT SALINE vs. WT KETAMINE: F(tr(1,20))=0.0216 p=0.886; F(time(2,20))=1.603 p=0.226; F(trx(time)(2,20))=0.202 p=0.818;
KO NOX2 SALINE vs. KO NOX2 KETAMINE: F(tr(1,12))=0.342 p=0.580; F(time(2,12))=2.134 p=0.161; F(trx(time)(2,12))=1.970 p=0.182;
WT KETAMINE vs. KO NOX2 KETAMINE: F(genotype(1,16))=0.0178 p=0.897; F(time(2,16))=2.947 p=0.081; F(trx(time)(2,16))=0.797 p=0.494;
WT KETAMINE vs. KO NOX2 SALINE: F(tr(1,16))=0.0709 p=0.797; F(time(2,16))=2.148 p=0.149; F(trx(time)(2,16))=0.430 p=0.658;
WT SALINE vs. KO NOX2 KETAMINE: F(tr(1,16))=0.0703 p=0.798; F(time(2,16))=0.854 p=0.444; F(trx(time)(2,16))=0.742 p=0.492;
WT KETAMINE vs. KO NOX2 SALINE: F(genotype(1,16))=0.1114 p=0.918; F(time(2,16))=2.965 p=0.080; F(trx(time)(2,16))=0.0328 p=0.968.

b) Mean of DA basal levels (fmol/20µl) ± SEM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SALINE</td>
<td>50.69 ± 0.78</td>
</tr>
<tr>
<td>WT KETAMINE</td>
<td>50.40 ± 0.38</td>
</tr>
<tr>
<td>KO NOX2 SALINE</td>
<td>50.45 ± 0.29</td>
</tr>
<tr>
<td>KO NOX2 KETAMINE</td>
<td>50.63 ± 0.12</td>
</tr>
</tbody>
</table>

Two way ANOVA for repeated measures:

WT SALINE vs. WT KETAMINE: F(tr(1,20))=0.0151 p=0.905; F(time(2,20))=1.188 p=0.325; F(trx(time)(2,20))=0.216 p=0.807;
KO NOX2 SALINE vs. KO NOX2 KETAMINE: F(tr(1,12))=0.0901 p=0.774; F(time(2,12))=0.190 p=0.829; F(trx(time)(2,12))=1.015 p=0.391;
WT KETAMINE vs. KO NOX2 KETAMINE: F(genotype(1,16))=0.0530 p=0.824; F(time(2,16))=0.694 p=0.514; F(trx(time)(2,16))=0.292 p=0.750;
WT KETAMINE vs. KO NOX2 SALINE: F(tr(1,16))=0.00189 p=0.496; F(time(2,16))=0.261 p=0.774; F(trx(time)(2,16))=0.906 p=0.424;
WT SALINE vs. KO NOX2 KETAMINE: F(tr(1,16))=0.00371 p=0.985; F(time(2,16))=0.489 p=0.622; F(trx(time)(2,16))=0.409 p=0.671;
WT SALINE vs. KO NOX2 SALINE: F(genotype(1,16))=0.00746 p=0.933; F(time(2,16))=0.441 p=0.651; F(trx(time)(2,16))=0.509 p=0.611.

c) Mean of DA basal levels (fmol/20µl) ± SEM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SALINE</td>
<td>53.48 ± 2.75</td>
</tr>
<tr>
<td>WT AMPHETAMINE</td>
<td>53.64 ± 0.50</td>
</tr>
<tr>
<td>KO NOX2 SALINE</td>
<td>52.2 ± 0.43</td>
</tr>
<tr>
<td>KO NOX2 AMPHETAMINE</td>
<td>56.91 ± 1.74</td>
</tr>
</tbody>
</table>

Two way ANOVA for repeated measures:
WT SALINE vs. WT AMPHETAMINE: $F_{(1,12)}=2.653$ $p=0.154$; $F_{(2,12)}=2.378$ $p=0.135$; $F_{txr(2,12)}=0.0175$ $p=0.983$; KO NOX2 SALINE vs. KO NOX2 AMPHETAMINE: $F_{(1,12)}=1.721$ $p=0.238$; $F_{(2,12)}=1.342$ $p=0.298$; $F_{txr(2,12)}=1.559$ $p=0.250$; WT AMPHETAMINE vs. KO NOX2 AMPHETAMINE: $F_{gen(1,12)}=0.330$ $p=0.587$; $F_{(2,12)}=0.0366$ $p=0.964$; $F_{txgen(2,12)}=0.158$ $p=0.856$; WT AMPHETAMINE vs. KO NOX2 SALINE: $F_{(1,12)}=0.997$ $p=0.357$; $F_{(2,12)}=1.867$ $p=0.197$; $F_{txr(2,12)}=1.217$ $p=0.330$; WT SALINE vs. KO NOX2 AMPHETAMINE: $F_{(1,12)}=2.425$ $p=0.170$; $F_{(2,12)}=3.266$ $p=0.074$; $F_{txr(2,12)}=0.176$ $p=0.841$; WT SALINE vs. KO NOX2 SALINE: $F_{gen(1,12)}=1.605$ $p=0.252$; $F_{(2,12)}=1.572$ $p=0.248$; $F_{txgen(2,12)}=0.318$ $p=0.733$.

d) Mean of Glu basal levels (µMol) ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SALINE</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>WT AMPHETAMINE</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td>KO NOX2 SALINE</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>KO NOX2 AMPHETAMINE</td>
<td>0.42 ± 0.01</td>
</tr>
</tbody>
</table>

Two way ANOVA for repeated measures:
Supplementary Figure 1 Behavioral alterations 4 hours after ketamine injection. Four hours after injection with ketamine (30 mg/kg, i.p.) or saline, mice were placed in the arena for open field test. Graphics represent the frequency of grooming (A), rearing (B), crossing (C) and sniffing (D) recorded during the 20 minutes of the test in wild-type (WT) and NOX2-deficient (KO NOX2) mice.
For grooming: F_{\text{treatment(tr)} \times \text{genotype(gen)}}(1,14)=5,500 \ p=0.034, \ F_{t(1,14)}=8.962 \ p=0.010, \ F_{\text{gen}(1,14)}=5,005 \ p=0.042; \ n.s.= \text{not significant}: \text{WT saline vs. KO NOX2 saline } p=0.943; \text{KO NOX2 saline vs. KO NOX2 ketamine } p=0.654. For rearing: F_{\text{treatment(tr)} \times \text{genotype(gen)}}(1,14)=6,482 \ p=0.023, \ F_{t(1,14)}=4.922 \ p=0.044, \ F_{\text{gen}(1,14)}=5.055 \ p=0.041; \ n.s.= \text{not significant}: \text{WT saline vs. KO NOX2 saline } p=0.845; \text{KO NOX2 saline vs. KO NOX2 ketamine } p=0.820. For crossing: F_{\text{treatment(tr)} \times \text{genotype(gen)}}(1,14)=7.258 \ p=0.017, \ F_{t(1,14)}=6.560 \ p=0.023, \ F_{\text{gen}(1,14)}=5.117 \ p=0.040; \ n.s.= \text{not significant}: \text{WT saline vs. KO NOX2 saline } p=0.776; \text{KO NOX2 saline vs. KO NOX2 ketamine } p=0.927. For sniffing: F_{\text{treatment(tr)} \times \text{genotype(gen)}}(1,14)=5.695 \ p=0.032, \ F_{t(1,14)}=7.302 \ p=0.017, \ F_{\text{gen}(1,14)}=7.493 \ p=0.016; \ n.s.= \text{not significant}: \text{WT saline vs. KO NOX2 saline } p=0.817; \text{KO NOX2 saline vs. KO NOX2 ketamine } p=0.837. **p<0.01: \text{WT ketamine vs. WT saline and WT ketamine vs. KO NOX2 ketamine using two-way ANOVA followed by Tukey post-hoc test } (n=4 \text{ WT saline}; n=5 \text{ WT ketamine}; n=4 \text{ KO NOX2 saline}; n=5 \text{ KO NOX2 ketamine}).
Supplementary Figure 2 NOX2 enzyme is expressed in the mouse prefrontal cortex. (a) RT-PCR for Nox2, its subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, $p22^{phox}$) and L32 mRNA in prefrontal cortex (PFC) and spleen of wild-type mice ($n=5$). Spleen was used as positive control since NOX2 is mainly expressed in this organ (Bedard and Krause, 2007). Reverse transcription negative control (RT-) contained experimental RNA, was proceeded in the same manner, but did not contain superscript enzyme. PCR negative control (PCR-) was obtained by substituting the reverse transcribed cDNA with water in the PCR reaction mixture. (b)
Western Blotting for NOX2 in PFC of two representative wild-type mice (n=4). Spleen and brain of wild-type (WT) and NOX2-deficient (KO) mice were used as positive and negative control, respectively.

**Supplementary Figure 3**

Parvalbumin mRNA does not diminish after acute ketamine exposure. Real-Time PCR quantification of parvalbumin mRNA in prefrontal cortex after saline or ketamine injection in wild type (WT) and NOX2-deficient (KO NOX2) mice. ns =not significant using two-way ANOVA (n=5). $F_{\text{gen}}(1,16)=0.0874, p=0.771$, $F_{\text{tr}}(1,16)=0.332, p=0.572$, $F_{\text{gen}}(1,16)=3.955, p=0.064$. 

Supplementary Figure 3
CONCLUSIONS AND PERSPECTIVES

We have analysed the role of NOX2 in two different animal models of schizophrenia. In a first study using the post weaning isolation in the rat, we have found a dramatic increase of NOX2 mRNA expression in specific brain regions. Although NOX2 expression has been reported in neurons, astrocytes and microglia, we failed to demonstrate which cell was responsible for this increase because of lack of specific antibodies. However the accumulation of microglia in the concerned brain regions suggests that NOX2 up-regulation accounts for increased microglial cells. The exact cellular localization of NOX2 by in situ hybridization should give an answer to this question. The fact that NOX2 and its subunits p22phox, p40phox, p47phox and p67phox mRNA levels are very high in isolated animals while they are hardly detectable in non isolated rats raises a number of questions and perspectives. On a mechanistic point of view, it could be useful to identify common signals and transcription factors enhancing the expression of NOX2 and subunits. Another approach would be to measure NOX2 expression by RT-PCR in brain samples from schizophrenic patients. This is a feasible approach as large libraries of brain material are available for neurochemistry. If similarly to our model, an increase of NOX2 expression is detected in the brain of schizophrenics, this would represent a strong hint for the development of NOX2 inhibitors as potential antipsychotic drugs. In the second study using ketamine administration in mice, we have observed that NOX2-deficient mice did not develop behavioural anomalies and that the increase of glutamate and dopamine was blunted. This finding as well as a decrease in NMDA receptor 2A provides a possible mechanistic link for a common role of NOX2 in the behavioural and biochemical alterations observed in the social isolation model. Future research should aim at understanding the mechanisms leading to NOX2 activation and more crucially how NOX2 is regulating glutamate and dopamine release. In particular, we have already undertaken studies assessing the effect of ketamine using i) mutant mice carrying a mutation in the Ncf1 gene leading to p47phox inactivation and diminished NOX2 activation, ii) the same mutant mice which have been genetically modified for a specific expression of a normal p47phox transgene in macrophages/microglia. If it is the absence of NOX2 in microglia
which is responsible for the lack of ketamine-induced response, these latter transgenic mice should develop the altered behaviour seen in control animals. These experiments should not only lead to a better understanding of the role of NOX2 in neural pathologies, but also may benefit to the design of novel strategies for the control of schizophrenia development.
GRAZIE A...

Tutto quello che si può leggere in queste pagine non sarebbe stato realizzabile senza il preziosissimo aiuto di tantissime persone.

Il mio primo immenso ed infinito ringraziamento è per mio marito Michele, per aver avuto l’immensa pazienza e la fortissima volontà di sopportare ed accettare i 1500 km che ci hanno diviso per 2 lunghi anni, per aver poi accettato di rivoluzionare interamente la sua vita e lasciare tutto ciò che aveva in Italia per seguirmi qui a Ginevra, per aver accettato di vivere in una realtà completamente diversa da quella a cui era abituato, per essermi stato sempre vicino, per aver condiviso con me ogni gioia e ogni momento difficile di questi anni, per avermi incoraggiata ad andare avanti anche quando le difficoltà sembravano insormontabili, per avermi ascoltata e per essere stato il mio “capro espiatorio” in tanti momenti bui. Ma soprattutto lo ringrazio per aver voluto costruire con me, nonostante le mille difficoltà “pratiche” che stiamo vivendo, la nostra meravigliosa famiglia.

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Ringrazio la Prof.ssa Trabace per avermi permesso di vivere questa esperienza del dottorato e per avermi dato l’opportunità di affrontare e confrontarmi con “la dura realtà” della ricerca italiana, spingendomi sin dall’inizio a spostarmi all’estero per poter crescere professionalmente e poter “aprire” al massimo la mia mente.

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