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Review

Neuronal nitric oxide synthase (*NOS1*) and its adaptor, *NOS1AP*, as a genetic risk factors for psychiatric disorders

F. Freudenberg[†], A. Alttoa[‡,§] and A. Reif[†,*]

[†]Department of Psychiatry, Psychosomatics and Psychotherapy, University Hospital of Frankfurt, Frankfurt am Main, Germany,
[‡]Department of Psychiatry, Psychotherapy and Psychosomatics, University Hospital of Würzburg, Würzburg, Germany, and
[§]Department of Psychology, Estonian Centre of Behavioural and Health Sciences, University of Tartu, Tartu, Estonia
**Corresponding author: A. Reif, Department of Psychiatry, Psychosomatics and Psychotherapy, University Hospital of Frankfurt, Heinrich-Hoffmann-Straße 10, 60528 Frankfurt am Main, Germany. E-mail: andreas.reif@kgu.de*

Nitric oxide (NO) is a gaseous transmitter produced by nitric oxide synthases (NOSs). The neuronal isoform (NOS-I, encoded by *NOS1*) is the main source of NO in the central nervous system (CNS). Animal studies suggest that nitrinergic dysregulation may lead to behavioral abnormalities. Unfortunately, the large number of animal studies is not adequately reflected by publications concerning humans. These include post-mortem studies, determination of biomarkers, and genetic association studies. Here, we review the evidence for the role of NO in psychiatric disorders by focusing on the human *NOS1* gene as well as biomarker studies. Owing to the complex regulation of *NOS1* and the varying function of NOS-I in different brain regions, no simple, unidirectional association is expected. Rather, the 'where, when and how much' of NO formation is decisive. Present data, although still preliminary and partially conflicting, suggest that genetically driven reduced NO signaling in the prefrontal cortex is associated with schizophrenia and cognition. Both *NOS1* and its interaction partner *NOS1AP* have a role therein. Also, reduced *NOS1* expression in the striatum determined by a length polymorphism in a *NOS1* promoter (*NOS1* ex1f-VNTR) goes along with a variety of impulsive behaviors. An association of *NOS1* with mood disorders, suggested by animal models, is less clear on the genetic level; however, NO metabolites in blood may serve as biomarkers for major depression and bipolar disorder. As the nitrinergic system comprises a relevant target for pharmacological interventions, further studies are warranted not only to elucidate the pathophysiology of mental disorders, but also to evaluate NO function as a biomarker.

Keywords: ADHD, biomarker, bipolar disorder, impulsivity, major depression, nitric oxide, NOS1AP, NO_x, polymorphism, schizophrenia

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Nitric oxide (NO) is an atypical, gaseous signaling molecule which is formed by the enzyme neuronal nitric oxide synthase (NOS-I), encoded by the *NOS1* gene. The target structures of NO involve the NO receptor enzyme soluble G-cyclase as well as a variety of proteins that are nitrosylated, and hence functionally changed due to this post-translational modification (Snyder & Ferris 2000). Downstream effects are manifold and involve activation of kinases as well as genomic effects, mediated, e.g. by CREB (Riccio *et al.* 2006). Thus, the precise mechanism of action regarding intracellular effects is mainly governed by the subcellular compartment where NOS-I is localized. NOS-I is a 1434-amino-acid protein that is composed of a C-terminal oxygenase domain that converts L-arginine to L-citrulline, resulting in the release of NO, and a reductase domain that donates electrons to the oxygenase domain by reducing NADPH to $NADP^+$ (Fig. 1a). NOS-I is widely expressed throughout the brain, especially in the cerebellum, the basal ganglia, hippocampus, frontal cortex and most other regions (Blum-Degen *et al.* 1999). The enzymatic activity of NOS-I requires dimerization of two NOS-I monomers and is dependent on binding of Ca^{2+} and calmodulin. Hence, NOS-I can be activated by a number of upstream signaling cascades; most prominently, NOS-I is physically coupled to the NMDA receptor complex via PSD-93/-95 (Brenman *et al.* 1996) at the post-synaptic density in glutamatergic neurons (Nedvetsky *et al.* 2002). Specifically, NOS-I carries a PDZ-domain that interacts with the PDZ2 domain of PSD-95 (and also PSD-93), anchoring NOS-I to the post-synaptic density (Doucet *et al.* 2012). The GluN2 subunits of NMDA receptors in turn bind to the PDZ1 or PDZ2 domain of PSD-95, bringing the NMDA receptor in proximity to NOS-I thereby allowing NMDA receptor-mediated Ca^{2+}-influx to activate and control NO production by NOS-I (Fig. 1b). Therefore, the NOS-I/PSD-95/NMDA receptor complex appears to be critical for the physiological integrity of NOS-I especially in the glutamatergic post-synapse (Doucet *et al.* 2012; Weber *et al.* 2014). In addition to the

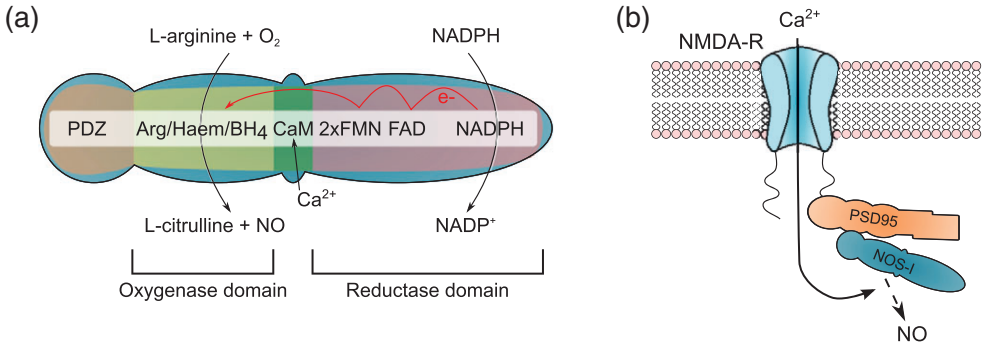

Figure 1: Structure and function of *NOS1*. (a) Structure of the NOS-I protein. (b) NMDA receptors (NMDA-Rs) and NOS-I both interact with PDZ domains of PSD-95. Through this close interaction NOS-I activation can be controlled by Ca^{2+}-influx through NMDA-Rs.

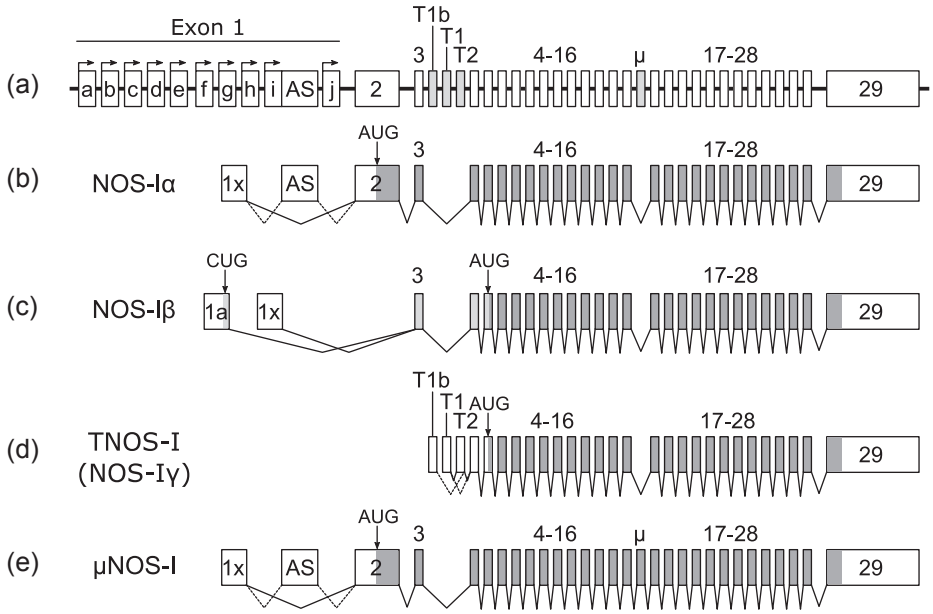

Figure 2: Structure of the human *NOS1* gene and mRNA variants. (a) Genomic organization of the human *NOS1* gene displaying all exons including alternative first exons (1a-1j, AS) and exons only expressed in testicle or skeletal muscle (T1b, T1, T2, μ; gray shading). The alternative first exons (1a-1j) are driven by individual promoters (indicated by the arrow). (b) Schematic structure of the NOS-Iα mRNA coding for the full-length protein. One of the alternative first exons is spliced to exon 2 or alternatively to AS, which in turn is spliced to exon 2. Translation starts in exon 2 (indicated by the AUG) and stops in exon 29 [the open reading frame (ORF) is indicated by the gray shading]. (c) The NOS-Iβ variant is lacking exon 2 (and also AS) and therefore does not code for the PDZ-domain and PIN-binding domain. Translation of the protein is initiated either at a CUG in exon 1a or, for all other first exons, an AUG in exon 5 (again the ORF is indicated by the gray shading, with the alternative exon 1a part of theORF shaded light gray). (d) The TNOS-I (NOS-Iγ) variant is only expressed in testicles and is missing exons 1–3. Protein translation for this variant starts at the AUG in exon 5. (e) The skeletal muscle specific variant μNOS-I contains all the exons of NOS-Iα plus the μ exon located between exon 16 and 17. Note that this figure is not true to scale.

interaction with PSD-93/-95, the PDZ domain of NOS-I interacts with NOS1AP (NOS-I adapter protein, the protein previously known as CAPON; see below). NOS1AP binding to NOS-I directly competes with the interaction between NOS-I and PSD-93/-95 and alters the subcellular localization of NOS-I (Jaffrey *et al.* 1998), allowing interaction of NOS-I with other proteins, including RASD1 (Fang *et al.* 2000) and SYN1 (Jaffrey *et al.* 2002).

Structure of the human NOS1 gene

The human *NOS1* gene has been mapped to chromosome 12q24.2-.31 (Boissel *et al.* 1998; Hall *et al.* 1994); it consists of 28 coding exons, distributed over 240-kb genomic DNA. A 130 kb 'variable region' harbors 12 alternative first exons along with unique promoters (Wang *et al.* 1999). These alternative first exons were termed exons 1a to 1l (Bros *et al.* 2006; Saur *et al.* 2002b; Wang *et al.* 1999; Fig. 2), and make

NOS1 – along with the glucocorticoid receptor – one of the most complex genes in the human genome (Zhang *et al.* 2004). The alternative first exons are not translated into protein and are expressed in a tissue- and/or cell-specific manner (Boissel *et al.* 1998). The biological role of these alternative first exons is unclear, but they may well serve to allow cell-specific expressional regulation.

There are several confusing nomenclatures regarding the alternative first exons 1a to 1l (Table 1). Initially, two alterative first exons were described and termed 5′1 and 5′2 (Xie *et al.* 1995); this terminology was later expanded to an exon called 5′3. As more alternative first exons were discovered, an alphabetical order was introduced (Wang *et al.* 1999). However, even this alphabetical order is misleading, as exon 1c according to Wang et al. as well as Saur and associates (Saur *et al.* 2002b) is designated as exon 1d by Förstermann's group (Bros *et al.* 2006). In contrast, the latter describe a hardly expressed exon [1c], while on the other hand they do not report a low-expressing exon [1e]. Notations from exons 1f up to 1h are identical across investigators. Exons 1i, 1j and 1k form a 700-bp region and show overlap; exon AS according to Wang *et al.* (1999) corresponds to exon 1k in Förstermann's classification (Bros *et al.* 2006; Table 1). In order to keep the consistency between this paper and our previous publications, we will use the nomenclature of Wang and colleagues, although consensus on an unambiguous system is a desideratum.

Alternative first exons are driven by eleven distinct promoters. Exons 1g and 1f are highly conserved, suggesting that they represent the evolutionarily ancient first exons. While these exons are merged and share one promoter in rodents, they are separated by 300 bp in humans and possess separate and distinctly regulated promoters (Rife *et al.* 2000; Xie *et al.* 1995). The alternative first exon is part of the 5′UTR and thus will not be translated into protein. Up to now, it is still unclear how the apparently complex transcription is regulated and which functional consequences it has. However, it is known that there is tissue-semispecificity of alternative first exons (Boissel *et al.* 1998; Bros *et al.* 2006; Saur *et al.* 2002b), i.e. some first exons are restricted to specific tissues, while others seem to be expressed by a wide variety of cells. On the other hand, more than one alternative first exon can be expressed in a given tissue. Both exons 1c and 1f/g are expressed in human brain (Saur *et al.* 2002b). There is solid evidence that exon 1c is expressed in human cortex and hippocampus, however, in contrast to Bros *et al.* (2006) we failed to detect significant levels of exon 1f in these structures (Reif *et al.* 2006a). This might be either due to dynamic regulation of this first exon, or due to differing expression of these exons in various sub-regions of these structures. Additionally, we demonstrated a pronounced expression of exon 1f in the striatum (Reif *et al.* 2006a). Together, these data indicate that the usage of alternative first exons allows an exceptional fine-tuning of *NOS1* expression by means of a variety of transcription factors, thereby regulating temporal and spatial *NOS1* transcription. This might play a role in the physiological regulation and distribution of NOS-I, but dysregulation at this level could be implicated in pathophysiological processes as well. For example, it was shown that downregulation of exon 1c in infantile hypertrophic pylorus stenosis was accompanied by presumably compensatory upregulation of exon 1f (Saur *et al.* 2004).

There is a considerable number of splice variants translated from the *NOS1* gene which have not been fully described yet: the several known splice variants of the gene are termed NOS-Iα, -β and -γ (Fig. 2; Saur *et al.* 2002a). NOS-Iα is the full-length transcript including a first exon, and the PDZ-domain coding exon. In addition, NOS-Iα can also contain an alternatively spliced exon (AS) between exons 1 and 2 resulting in reduced levels of protein expression (Newton *et al.* 2003). The β-variant differs in the organization of the first exons, lacking the second (and sometimes the third) exon harboring the PDZ- and PIN-binding domains (Brenman *et al.* 1997). As a consequence, the resulting soluble protein is differentially trafficked due to the missing PDZ–PSD-95 interaction necessary for membrane association. The same is true for the N-terminally truncated, testis-specific transcript which is termed TNOS-I (NOS-Iγ; Brenman *et al.* 1997) and driven by testis-specific promoters linked to alternative first exons between exons three and four (Wang *et al.* 1997). Cassette deletions of exons 9 and 10, again of unknown significance yet with probable expression during synaptogenesis were also observed (Ogilvie *et al.* 1995). Finally, two short transcripts of unclear function were additionally detected, NOS-002 (which comprises alternative exons 1a and 2), and NOS-003, which features an additional short exon ('μ', specific for skeletal muscle) interposed between exons 16 and 17 forming a transcript of those three exons. This μ-exon is also found as a cassette insertion in full-length NOS-I in skeletal muscle (μNOS-I).

Genetic variants of NOS1

Owing to the size of the coding region, several non-synonymous exonic single nucleotide polymorphisms (SNP) are deposited in the databases, none of which, however, has conclusively shown to be functional. In the promoter region of exon 1c, a SNP has been described (*NOS1* ex1c-SNP, rs41279104) which reduces the expression of this exon by 30% if the minor allele is present (Saur *et al.* 2004) and which we also confirmed to affect brain expression of *NOS1* in humans (Weber *et al.* 2014). Three microsatellites have been described in greater detail: an intronic AAT repeat of unclear significance, which was found to differ in allele frequency in three populations (Grasemann *et al.* 1999); a CA repeat in the 3′UTR of exon 29 was already described in the first description of the human *NOS1* gene and suggested to impact on *NOS1* mRNA processing (Hall *et al.* 1994); and a highly polymorphic CA repeat 33 bp upstream of the TATA box of exon 1f termed *NOS1* ex1f-VNTR, which varies between 180 and 210 bp in length. Although the latter repeat appears to be present in other mammalian species, it seems to be much shorter and not as polymorphic: initial data from 20 rhesus monkeys demonstrated that repeat length variation only differs by maximally two nucleotides (unpublished data). An intriguing feature of this repeat is its apparent non-random distribution with clustering at the alleles 182/184, 192 and 200/202/204, i.e. short, intermediate and long alleles. By conducting reporter gene assays, we confirmed functionality of this repeat as expressional activity

Table 1: Differing nomenclatures of alternative first exons

Numerical			5′3			5′2				5′1		
Wang, Saur	a	b	—	c	e	f	g	h	i	—	AS	j
Föstermann	A	B	C	D	E	F	G	H	I	J	K	L

is enhanced in longer repeats (Reif *et al.* 2006a, 2009). This finding was then confirmed by another group (Rife *et al.* 2009) using constructs of size comparable to our study, thus, the evidence for the molecular functionality of this polymorphism is quite compelling. Furthermore, transcriptional changes of human BA46 were detected as a function of *NOS1* ex1f-VNTR. Meaningfully dysregulated genes included α-synuclein, *RGS4*, and, interestingly, *GRIN1* – the gene encoding subunit 1 of the NMDA receptor, i.e. the direct upstream activator of NOS-I. Having established functionality of the repeat and clustering of the repeat in discrete islands, we dichotomized the repeat in following studies into short (180–196 repeats) and long (198–210 repeats) alleles to facilitate further genetic studies. By doing so, no differences in short vs. long repeats were found in control populations from Germany, Estonia, Austria, Norway, Sweden, Spain and Italy. However, significantly different values were obtained from patient populations (see below). Interestingly, longer repeats were shown to result in higher levels of exhaled NO metabolites, further supporting the functional role of this polymorphism (Texereau *et al.* 2004).

Data from animal studies

Animal studies, carried out mainly in mice and rats and using genetic as well as pharmacological approaches, argue for a multifaceted role of NO in the regulation of behavioral traits. Many behavioral domains are influenced by NO: impulsivity and aggression (Nelson *et al.* 2006), exploratory vs. anxious behavior, depression-like symptoms, as well as cognitive performance (Wultsch *et al.* 2007) were all shown to be influenced by manipulating NO levels. This multitude of effects cannot be explained in a simplistic way. Rather, localization and timing of NO production appears to be crucial for its effect on behavior: the 'where, when and how much' of NO formation is decisive for the subsequent downstream effect, especially as the NO system is widespread and loose as compared to the clearly defined monoamine neurotransmitter systems. Also, there is no defined neuroanatomical structure producing NO in a centralized manner, but rather every NO-producing neuron is on its own and synthesizes NO in a de-synchronized way (Kiss & Vizi 2001). In addition, there are no 'nitrinergic tracts'; rather, NO acts in a local, non-synaptic and diffusion-controlled field around its site of generation thereby potentially targeting the post-synaptic neuron (where NO then acts as a second messenger), as well as surrounding neurons including the pre-synaptic neuron and glial cells. Given the various effects NO exerts in different cell types, it can easily be envisaged that NO has different effects in different neuroanatomical structures, which might even be antagonistic on the behavioral level. Clearly, when interpreting data on NO effects, it is crucial to specify neuroanatomical region, cell type, and downstream effects.

Soon after NO had been identified as an important moderator of behavior in rodents, its contribution to human brain function and disease was studied. Human investigations are hampered by several factors: (1) NO as such does not cross the blood–brain barrier, so that it can neither be administered nor measured in the periphery, (2) there are only very limited possibilities to influence NO levels in the brain, and (3) there is no possibility to directly measure NOS-I activity in the human brain. Thus, studies have to rely on indirect measurements, comprising post-mortem studies and genetic approaches.

The relation of the *NOS1* gene to psychiatric disorders and phenotypes

Being such a large gene, it is not surprising that *NOS1* features a complex haplotype structure. According to HapMap data, *NOS1* is composed of at least 16 haploblocks. As calculated by Tagger, 300 tagging SNPs have to be genotyped to assess the complete haplostructure. Therefore, it is not surprising that early studies, which investigated only a single and synonymous or intronic SNP, failed to detect an association of *NOS1* with various disease states (see below). Thus, association and other genetic studies have to be interpreted in the light of the complex genetic architecture of *NOS1*.

Given the pleiotropy of NO with respect to brain regions, inter- and intracellular functions, interactions with signaling pathways and upstream activators, it is not surprising that there is no single, straightforward molecule-to-disease connection. Rather, NO is implicated in a wide range of neuropsychiatric disorders, and partially different mechanisms of NO contributing to disease are operative. Nitrinergic signaling at the synaptic machinery underlying the NMDA synapse and interactions between NO and dopamine in the striatum might be crucial in schizophrenia and possibly bipolar disorder. The close link between the serotonergic and nitrinergic systems likely plays an important role in depression and anxiety. The link between NO and impulsive behaviors might well be due to both of these interactions. In contrast, the contribution of NO to neurodegenerative disorders seems to involve increased oxidative stress but also glutamate excitotoxicity. Thus, NO – similarly to 5HT – seems to be a master control molecule serving manifold functions and consequently contributing to many different pathologies. Accordingly, plenty of phenotypes have been linked to *NOS1* – from stroke susceptibility (Manso *et al.* 2012), Alzheimer's dementia (Galimberti *et al.* 2007), Parkinson's disease (Rife *et al.* 2009), restless legs syndrome (RLS; Winkelmann *et al.* 2008) to 'soft' phenotypes such as aging and longevity (Montesanto *et al.* 2013) or psychological distress (Luciano *et al.* 2012). However, the largest share of the studies were dedicated to three symptom clusters: schizophrenia and cognition, impulsivity and

attention deficit/hyperactivity disorder (ADHD), and depression and anxiety.

Schizophrenia

A tentative link between NO metabolism and schizophrenia was first made in Russia in the 1960s (Averbukh *et al.* 1966), but it was not until the early 1990s that work began in detail. Histochemists had introduced NADPH diaphorase histochemistry as a tool to label neuronal populations expressing NOS (Vincent *et al.* 1982) and the importance of NO was beginning to sink in – it was even named 'molecule of the year 1992'. Post-mortem studies (Bernstein *et al.* 2011b) were mainly carried out in schizophrenia, and, to a lesser extent, affective disorders. Bearing in mind the manifold problems that accompany post-mortem studies, in combination with usually small sample sizes, results were – not unexpectedly – quite heterogeneous. Overall, human data supporting a role of NO in the pathophysiology of schizophrenia have found both increases and decreases in NO levels (Bernstein *et al.* 2005). A possible explanation for the conflicting data might well be that NOS activity could be upregulated in one brain region and downregulated in another region at the same time (Bernstein *et al.* 2011a,2011b). The latter reviews also provide a scholarly overview on NOS-I pathohistology in schizophrenia and are highly recommended for further reading. Taken together, synthesizing all available data, qualitative as well as quantitative abnormalities of NOS-I-positive neurons in the frontal cortex, basal ganglia and other brain regions were found in schizophrenia.

Evidence for an involvement of the *NOS1* gene in psychoses exists since the early days of psychiatric molecular genetics, when linkage studies supported a locus for endogenous psychoses on chromosome 12q22-24, the region harboring *NOS1*. Linkage signals were found in major depression, bipolar disorder (replicated eight times) and schizophrenia (as summarized and referenced in Fig. 1 in Reif *et al.* 2006a). Interestingly, a single marker within *NOS1* itself was significantly linked to bipolar disorder, while D12S366, located only 800 kb from *NOS1*, was found to be associated with bipolar disorder and schizophrenia in three studies. Together, these early linkage analyses suggested *NOS1* as a promising positional candidate gene.

As *NOS1* can also be considered a functional candidate gene for its interactions with the glutamatergic and the dopaminergic systems, it was soon subjected to candidate gene based studies. Of those, one study (Fallin *et al.* 2005) conducted in Ashkenazi Jews, employed a family-based design and yielded positive results. The first case–control study, however, was published in 2002 and tested a potentially functional SNP in the 3′UTR in a sample of 215 Japanese schizophrenic patients (Shinkai *et al.* 2002), again with positive outcome. Subsequently, our group conducted a mutation analysis, qRT-PCR and haplotype analysis in Caucasian patients suffering from schizophrenia, suggesting that the functional promoter SNP rs41279104 – resulting in decreased gene expression (Saur *et al.* 2004; Weber *et al.* 2014) – is associated with disease (Reif *et al.* 2006a). Since then, six further case–control association studies on schizophrenia and *NOS1* were published in total (Cui *et al.* 2010; Nicodemus *et al.* 2010; Okumura *et al.* 2009; Riley *et al.* 2010; Tang *et al.* 2008; Wang *et al.* 2012). Four of those came from Chinese and Japanese populations, with mixed results: while Cui and associates replicated the positive finding on rs41279104 (and also provided evidence for reduced NOS-I expression on the protein level in BA9 – part of the dorsolateral prefrontal cortex – for risk allele carriers), Okumura did not, although two other *NOS1* SNPs were significant. The same was true for the study by Wang in China, where a SNP in intron 2 of *NOS1* was nominally significant. In contrast, the other Chinese study found significant evidence for an association of *NOS1* (5′UTR and intron 2) with schizophrenia as well. In an Irish sample no association of *NOS1* with schizophrenia was shown. However, only four SNPs were tested and did not include the previously significant rs41279104 (Nicodemus *et al.* 2010). Finally, a CA repeat in the 3′UTR of exon 29 was not associated with schizophrenia in another Asian sample (Liou *et al.* 2003). Taken together, these association studies rather argue for an association of the 5′ end of *NOS1* – especially the promoter region – with schizophrenia (Fig. 3). In order to corroborate this assumption, we extended our initial study and conducted a meta-analysis of published case–control studies (Weber *et al.* 2014) which confirmed a significant association of the *NOS1* promoter SNP rs41279104 (odds ratio of 1.29, $n = 1526$ cases). We could also show that this SNP influences not only reporter gene expression in heterologous cell systems, but indeed also *NOS1* expression in human prefrontal cortex as measured in post-mortem brain samples. Reduced methylation of the *NOS1* promoter as found in a genome-wide epigenetic study in schizophrenia (Wockner *et al.* 2014) might well represent a compensatory mechanism to counteract genetically driven reduced *NOS1* expression in the prefrontal cortex. However, it has to be mentioned that a small post-mortem study did not provide evidence for reduced *NOS1* expression in the dorsolateral PFC (Silberberg *et al.* 2010), but on the contrary, exons 1c and 1f were overexpressed. Thus, further and larger studies are needed to clarify where and how *NOS1* expression levels differ in schizophrenia.

Not only linkage analysis and candidate gene studies suggested *NOS1* as a risk gene for schizophrenia, but also hypothesis-free genome-wide association studies (GWAS) were in line with this assumption. The first GWAS arguing for a role of *NOS1* in schizophrenia was already published in 2008 in 7308 cases (O′donovan *et al.* 2008), with the SNP in *NOS1* reaching rank #6. The latest genetic research strategy relies on mutational screening using deep sequencing. In an initial study focusing on the exons coding for the catalytic domain, we could not identify rare mutations in patients (Reif *et al.* 2006a) which was not surprising given the low number ($n = 160$) of included subjects. However, a second scan in another 20 patients revealed a private missense mutation (G3608A) causing an exon 23 R1203H amino-acid substitution, which is predicted to be benign (PolyPhen online tool, http://genetics.bwh.harvard.edu/pph/index.html), in a schizophrenic patient who later committed suicide. This mutation could not be detected in more than 500 other cases and controls (unpublished data). The precise role of this mutation remains to be established, as no segregation data could be obtained. There are deep sequencing studies in *NOS1*

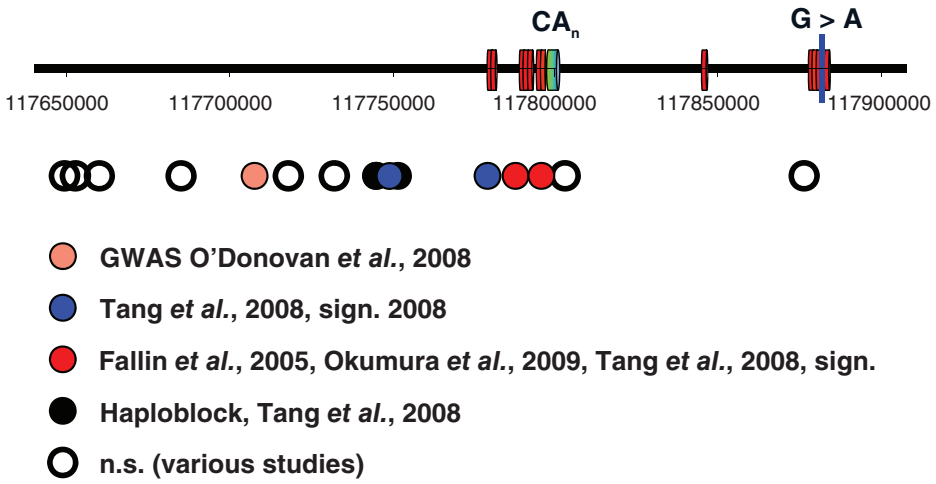

Figure 3: Overview on the localization of *NOS1* SNPs tested for an association with schizophrenia in relation to alternative first exons of *NOS1* (red) and chromosomal position.

under way in several laboratories at the moment; however, data has not yet been made available to the public.

Functional genomics of NOS1 in schizophrenia and executive functioning

The top hit regarding the *NOS1* gene from the O'Donovan study, rs6490121, was followed up in further studies and shown to be associated with working memory, as homozygous carriers of the risk allele performed more poorly (Donohoe *et al.* 2009). This effect was also observed for verbal IQ measures. That same SNP was found to be associated with lower P1 visual evoked potentials elicited by a spatial working memory task in a high density EEG study (O'donoghue *et al.* 2012). Carriers of the risk allele showed significantly lower P1 responses than non-carriers, pointing to a function of *NOS1* even in early sensory processing. Finally, Rose *et al.* (2012) used voxel-based morphometry and showed that gray matter volume in the ventromedial prefrontal cortex is significantly reduced in risk allele carriers. They also conducted a spatial working memory test and demonstrated increases in the activation of frontoparietal working memory networks and a failure to disengage regions of the default-mode network for risk allele carriers. Adding to this data, we could show that short *NOS1* ex1f-VNTR repeats also impacted on prefrontal functioning (as tested with event-related potentials recorded during a continuous performance task) in schizophrenic patients demonstrating a functional role for this gene variant (Reif *et al.* 2006a), and emphasizing the functional effects of *NOS1* and this polymorphism on neural systems. This was later corroborated by respective studies in healthy control subjects (see below). In our group, we performed a genomic imaging study using functional near-infrared spectroscopy (fNIRS) and an n-back paradigm to probe working memory. We found significant load-associated oxygenation differences between the genotype groups in the dorsolateral prefrontal and the parietal cortex. Specifically, short (i.e. risk) allele carriers showed a significantly larger increase in oxygenation in all load conditions. This suggests a potential compensatory mechanism, with task-related brain regions being more active in short-allele carriers to compensate for reduced *NOS1* expression (Kopf *et al.* 2011). Finally, the *NOS1* exon 1c rs41279104 risk allele was associated with slower reaction time in a working memory task, as well as with reduced right-hemispheric activation of the frontal cortex in the verbal fluency task (Reif *et al.* 2011c) further arguing for a role of *NOS1* in prefrontal functioning.

Taken together, present data strongly suggests that genetic variation in *NOS1* – mainly underlying reduced prefrontal expression of the gene –contributes to the genetic liability toward schizophrenia and may lead to compromised cognitive functioning and differential prefrontal brain activity also in healthy individuals. This might underlie the association with schizophrenia, as cognitive deficits are among the core symptoms of the disease. A tentative hypothesis how NOS-I might be implicated in schizophrenia pathophysiology suggests that short *NOS1* ex1f-VNTR alleles, presumably reducing expression of exon 1f, result in ineffective non-synaptic recruitment of neuronal assemblies in the striatum. This in turn might contribute to a dysfunctional dopaminergic output of the striatum (West *et al.* 2002) underlying increased impulsivity as well as defective response control found in schizophrenia. In contrast, exon 1c is expressed at high levels in the hippocampus, where NO functions as the second messenger of the NMDA receptor. By reducing exon 1c expression due to the presence of the risk allele, *NOS1* ex1c-SNP thus might contribute to hypoglutamatergic states known to be part of schizophrenia pathophysiology (Moghaddam 2003). As exon 1c is also predominantly expressed in the frontal cortex, genetically driven decreased *NOS1* expression in this region most likely results in compromised prefrontal NO signaling implicated in cognitive deficits in schizophrenia as well.

Clinical findings on NO and schizophrenia remain very sparse. One very small ($n = 8$), open study tested the soluble

G-cyclase inhibitor methylene blue as an adjuvant treatment in schizophrenia and found evidence for a modest improvement in psychopathology (Deutsch *et al.* 1997), an effect which has not been re-tested thus far; anecdotal observations did not support this finding (Turner 1985). In bipolar disorder, however, methylene blue – which was proposed as a 'calming agent' in psychiatric patients more than 100 years ago – was successfully used in a case report (Thomas & Callender 1985) and small studies probing acute (Narsapur & Naylor 1983; $n = 19$) and prophylactic (Naylor *et al.* 1986; $n = 31$) treatment regimens. Also, methylene blue's positive effects were reported in the acute treatment of bipolar depression (Naylor *et al.* 1987), but not mania (Naylor *et al.* 1988). However, as the action of methylene blue is very unspecific, and may also be attributable to inhibition of haeme-containing enzymes other than those related to the NO pathway, further clinical trials with selective inhibitors of NOS-I are needed. An alternative approach is the inhibition of NO production by way of overloading the L-arginine transport system with L-lysine. Indeed, in a pilot study, this strategy proved to have beneficial effects in schizophrenia (Wass *et al.* 2011). However, bearing in mind the above hypothesis that reduced *NOS1* expression in the prefrontal cortex contributes to schizophrenia, these findings are counterintuitive and prompt questions whether rather increased NO production might be beneficial in schizophrenia. Intriguingly, a recent study indeed demonstrated that administration of an NO-donor immediately and enduringly alleviates schizophrenia symptoms in patients (Hallak *et al.* 2013); however, this study still awaits replication which is urgently needed given the limitation of the study that NO does not cross the blood–brain barrier.

NOS1 adaptor protein (NOS1AP, CAPON), other NOS-I interaction partners and the genetic risk toward schizophrenia

In addition to the essential binding of calmodulin/Ca^{2+}, NOSs are characterized by highly intricate direct protein–protein interactions. The binding of NOS-I to its interaction partners is thought to either traffic the enzyme to specific subcellular components, to link it to upstream activators or downstream mediators or to regulate NOS-I activity. A closer look at the interaction partners of NOS-I is warranted, as many of them have also been proposed to play a role in psychiatric disorders.

NOS-I, in contrast to the other NOS isoforms, harbors a PDZ domain enabling binding to a wide range of protein ligands which feature the required binding motif (Jemth & Gianni 2007). By means of this domain, NOS-I binds to α1-synthrophin (in the skeletal muscle) and NOS1AP (NOS-I adaptor protein, previously termed CAPON: C-terminal PDZ-domain ligand of neuronal NOS), and by unique PDZ–PDZ domain interactions to PSD-93 and PSD-95. PSD-93/-95 proteins provide proximity of NOS-I to NMDA receptors (Brenman *et al.* 1996; Christopherson *et al.* 1999), so that on one hand, NOS-I is activated in an activity-dependent manner (by Ca^{2+} influx through NMDA receptors). On the other hand, the proximity of NO production to the NMDA receptor following NOS-I activation results in nitrosylation of the receptor, thereby inactivating it and providing negative feedback. By binding to PSD-93/-95, which feature three PDZ domains, the NO receptor sGC can interact with the NOS-I/PSD 95/NMDA receptor complex (Russwurm *et al.* 2001; Zabel *et al.* 2002), which links the site of NO production to its immediate effector site. The NOS-I/PSD 95/NMDA receptor complex is critical for physiological NOS-I function and dysfunction of this complex is believed to be involved in the development of psychiatric disorders including schizophrenia (Doucet *et al.* 2012; Weber *et al.* 2014; Zhou & Zhu 2009). Interestingly, elevated levels of *NOS1AP* were found in post-mortem tissues of patients with schizophrenia (Brzustowicz 2008) suggesting that disruption of NOS-I <> PDZ-interaction with PSD-93/-95 might have important implications for NOS-I's involvement in this disorder.

NOS1AP competes with PSD-93/-95 for NOS-I binding (Jaffrey *et al.* 1998) and hence alters subcellular localization of NOS-I; it features both a PDZ as well as an N-terminal phosphotyrosine binding (PTB) domain which allows it to connect NOS-I to synapsin, forming a ternary NOS-I–NOS1AP–synapsin complex (Jaffrey *et al.* 2002), and Dexras1 (Fang *et al.* 2000). The latter belongs to the superfamily of small GTPases and is itself activated by NO (Fang *et al.* 2000; Jaffrey *et al.* 2002), which is accomplished upon NOS1AP binding resulting in S-nitrosylation of a cysteine residue.

There is strong evidence that *NOS1AP* is a risk gene for schizophrenia (Brzustowicz 2008), as its locus on 1q21-q22, which is 700 kb apart from *RGS4*, is one of the major linkage hot spots for schizophrenia (Brzustowicz *et al.* 2000). By fine-mapping, *NOS1AP* was identified as the positional candidate at this locus (Brzustowicz *et al.* 2004), although a recent study analyzing a large British case–control sample implicated the nearby *UHMK1* gene instead (Puri *et al.* 2007, 2006). As *NOS1AP* is not only a positional, but also an attractive functional candidate gene for psychosis, several further association studies aimed to investigate this gene. Indeed, a study from Columbia examining microsatellites in 110 trios provided putative evidence for association (Miranda *et al.* 2006), in line with a large Chinese case–control sample interrogating nine tagging SNPs (Zheng *et al.* 2005). The latter, however, could not be confirmed in a recent family-based study of the same group (Fang *et al.* 2008). In a smaller study on 270 patients with schizophrenia we also found a significant association on the single marker level with disease (Reif et al., in prep: best *P*-value for rs945713, $P = 0.0072$). A small study from South America confirmed an association of *NOS1AP* with schizophrenia (Kremeyer *et al.* 2009), and recently, a non-coding but functional variant (rs12742393) affecting gene expression was shown to be associated with disease (Wratten *et al.* 2009). While meta-analyses are still lacking, these genetic studies gain impact by a recent paper demonstrating that *NOS1AP* expression is increased in schizophrenia and bipolar disorder; gene expression was also associated with at-risk genotypes (Xu *et al.* 2005). Finally, another recent study (Lencz *et al.* 2007) further supported the association of *NOS1AP* with schizophrenia. Consistent with this, two further studies demonstrated that *NOS1AP* is overexpressed in the prefrontal cortex of schizophrenic patients (Hadzimichalis *et al.* 2010; Xu *et al.* 2005). Mechanistically,

increased NOS1AP expression was suggested to compete with NOS-I binding to the PSD-95/NMDA receptor complex, thereby sequestering NOS-I and reducing NO signaling (Eastwood 2005). An authoritative review on NOS1AP and schizophrenia underscoring the relationship between *NOS1AP* and schizophrenia was recently provided by Linda Brzustowicz (2008) and is recommended for further reading. Apart from the association with schizophrenia, two rare non-synonymous variations were shown to segregate with obsessive compulsive disorder (OCD) and autism spectrum disorder (ASD; Delorme *et al.* 2010), respectively, arguing for a pleiotropic effect of NOS1AP on psychiatric phenotypes.

Neurodegenerative and movement disorders

As outlined above, NO contributes to oxidative stress pathways by its reaction with superoxide to form the extremely reactive peroxynitrite (ONOO−). The latter can cause lipid peroxidation and DNA damage ultimately leading to cell death due to energy depletion, but also nitrosylate tyrosine residues resulting in nitrotyrosine. Evidence for the contribution of NO to Alzheimer's dementia (AD) came, besides others, from a study (Luth *et al.* 2002) where nitrotyrosine staining was detected in both astrocytes as well as neurons in the brains from AD cases; in the latter cells, this was paralleled by aberrant NOS-I expression. A comprehensive review on the topic of NO's cytotoxic properties in the context of AD has been provided by Law and associates (Law *et al.* 2001). On the other hand, a selective and severe loss of NOS-I positive neurons in the hippocampus was evidenced by *in situ* hybridization, NADPH diaphorase (Norris *et al.* 1996) and NOS-I immunohistochemistry (Thorns *et al.* 1998), contradicting older studies arguing for a relative sparing of NOS-containing neurons in AD (Hyman *et al.* 1992; Mufson & Brandabur 1994). Thus, it might be speculated that initially raised NO levels (by means of oxidative stress) lead to the neuronal death of these neurons, resulting in a breakdown of NO-dependent memory formation in hippocampal circuits. This might be further aggravated by β-amyloid induced downregulation of the NO/cGMP/cGK/CREB pathway (Puzzo *et al.* 2005) or endocytosis of the NMDA receptor, mediated by dissociation of PSD-95, thus disrupting the NMDA/PSD-95/NOS-I/sGC protein complex (Snyder *et al.* 2005). As a clinical consequence, cognitive deficits typical for Alzheimer's disease are promoted, i.e. impairments of the transfer of the working memory content into long-term memory engrams. Most interestingly, analogous memory deficits in rats (generated by acetylcholine depletion) could be reversed upon administration of the novel nitrate GT 1061 (Bennett *et al.* 2007) which acted primarily in the hippocampus to activate sGC. Thus, the NO pathway is an attractive target in the treatment of cognitive deficits, and respective phase 1a studies with GT 1061 were conducted, but suspended due to hypotensive episodes. Delayed-release formulations of GT 1061 might overcome this problem; however, the compound is not developed further at present.

Intriguingly, linkage of late onset AD with the *NOS1* locus 12q22 was shown (Liang *et al.* 2006) and subsequently the 3′UTR C276T SNP of the *NOS1* gene was identified as a risk factor for AD (Galimberti *et al.* 2005), whereas the dinucleotide polymorphism in the 3′UTR of *NOS1* is not associated with disease (Liou *et al.* 2002). Further studies provided evidence that *NOS1* ex1f-VNTR short alleles significantly increased the risk toward development of AD (OR = 1.5) in a gene × gene interaction manner with the ApoE ε4 risk allele; presence of both risk alleles resulted in a more than 10-fold increased risk to suffer from AD (Galimberti *et al.* 2007). We have followed up this finding and could replicate ($P = 0.009$, corresponding to an OR of 1.77 (95% CI: 1.04–3.01)) the association of short alleles with Alzheimer's in an independent, cross-sectional sample from Vienna (the so-called VITA study). Again, an interaction with ApoE ε4 was observed (Reif *et al.* 2011a).

In Parkinson's disease, *NOS1* ex1f-VNTR was shown to be associated with disease in a very small ($n = 64$) Chinese sample (Lo *et al.* 2002); a significantly larger French study on 209 cases found evidence for an association of the 3′UTR C276T SNP (Levecque *et al.* 2003). A further study also suggested an association of eight (from 27) *NOS1* SNPs with Parkinson's disease; here, gene × environment interactions were also investigated. Yet, this did not result in overtly positive findings (Hancock *et al.* 2008). *NOS1* might contribute to the pathophysiology of Parkinson's disease either by promoting parkin S-nitrosylation (Chung *et al.* 2004; Yao *et al.* 2004), which has an important role in Parkinson's disease (Chung *et al.* 2005), or by influencing the dopaminergic tone of the basal ganglia (West *et al.* 2002). Intriguingly, short alleles of *NOS1* ex1f-VNTR (which is highly expressed in the striatum) has been shown to be associated with Parkinson's disease in two independent studies (Lo *et al.* 2002; Rife *et al.* 2009).

Most interestingly, a recent very comprehensive study identified *NOS1* as a positional candidate gene for RLS (Winkelmann *et al.* 2008). They applied a three stage design by first conducting an explorative case–control study in 367 cases using >1500 SNPs encompassing a 21-mb large linkage region on chromosome 12q23.1–12q24.31. The most significant SNPs were further explored in a second, independent sample of 551 cases. As the most significant SNP, rs7977109, was within *NOS1*, this gene was fine-mapped interrogating another 29 tagging SNPs in both samples combined; ten of those were significantly associated with RLS (and three of which survived correction for multiple testing) thereby strengthening the association finding. However, the precise genetic variants have yet to be identified. Unfortunately, the study did not include *NOS1* ex1f-VNTR, but it should be highlighted that the closest significant SNP is located only 4 kb downstream of *NOS1* ex1f-VNTR and that one of the only two SNPs which were significant in both stages is located 16 kb upstream, i.e. both SNPs flank the alternative first exons 1f/g rendering it possible that the causative genetic variant is localized in their vicinity. Thus, there is still a long way from genotype to phenotype; this journey, however, will most likely yield valuable insights not only into NO signaling, but also into the pathophysiology of neurodegenerative disorders in general.

Impulsivity and ADHD

Owing to data from *Nos1* knockout mice, which were more aggressive and impulsive (Nelson *et al.* 2006), a possible connection between *NOS1*, impulsive behaviors and related

phenotypes was soon explored on the human genetic level as well. Again, both genome-wide approaches as well as candidate gene based experiments supported a role of *NOS1* herein. The initial study on this phenotype came from our laboratory (Reif *et al.* 2009) and investigated *NOS1* ex1f-VNTR due to the strong expression of the corresponding alternative first exon in the basal ganglia. Carriers of the short allele presumably have decreased *NOS1* expression in the basal ganglia and the hippocampus. Since this functional characterization of this polymorphism, approximately 10 000 healthy controls and 5000 cases (various mental disorders) were genotyped in our laboratory. Consistently, short alleles of this polymorphism were linked to impulsive behaviors including adult ADHD, suicide, aggression and impulsive personality dimensions (Reif *et al.* 2009). In collaboration with colleagues from Estonia, we could show that risk alleles interact with early environmental factors to increase the likelihood to develop later-life maladaptive impulsivity (Reif *et al.* 2011b). Interestingly, in a separate sample also from Estonia, we could extend these findings to show that platelet MAO activity – presumably reflecting central MAO activity – moderates the effect of *NOS1* ex1f-VNTR on impulsivity: especially adaptive impulsivity was only increased in individuals having 'normal' MAO activity (Laas *et al.* 2010). Investigating the neural correlates of impulsive behavior in relation to NO, a neuroimaging study provided compelling evidence that the risk genotype goes along with increased impulsivity in a delay discounting paradigm in both adult ADHD as well as healthy controls, and that they displayed higher ventral striatal activity in this task (Hoogman *et al.* 2011). Other SNPs in the *NOS1* gene were associated with inattention in childhood ADHD (rs478597, $P = 8.08E{-}06$; Franke *et al.* 2009; Lasky-Su *et al.* 2008), as well as extraversion in a GWA study (Luciano *et al.* 2010). Owing to the involvement of the basal ganglia (especially the striatum and the subthalamic nucleus) not only in psychiatric aspects of impulsivity, but also in motor impulsivity (Volkmann *et al.* 2010), it makes sense that both the *NOS1* ex1f-VNTR as well as *NOS1* SNPs were associated with respective phenotypes (Reif 2010), i.e. Parkinson's disease and RLS (see above), which strengthens the notion that nitrinergic tone regulates striatal output.

In order to investigate the neurobiological mechanisms underlying the association findings, we conducted a Continuous Performance test with event-related potentials recorded in parallel in 167 healthy volunteers. Paralleling our findings in schizophrenic patients, short alleles were associated with decreased frontal brain activation while subjects performed the task. Using a topographical mapping method, the anterior cingulate cortex (ACC) was identified to be the neural structure responsible for this hypoactivation. *NOS1* thus seems to lead to increased impulsivity by affecting the ACC (Reif *et al.* 2009). As the ACC is involved in the detection of effort/reward correlations, it comprises an 'outcome monitoring system'. Genetically driven hypoactivation of the ACC might therefore bias the individual to impaired response toward long-term consequences, predisposing to immediate and impulsive acts. This might also lead to diminished goal-oriented and conscientious behavior which is reflected by the respective personality domains. Whether this hypothesis holds true has to be shown by further studies using all other neuroimaging modalities.

In a follow-up study, we recruited 300 healthy volunteers to undergo a neuropsychological test battery consisting of an n-back task, verbal and spatial memory tasks. Preliminary data on the effect of *NOS1* ex1f VNTR demonstrated that, in the n-back task, homozygous long-allele probands made significantly less commission errors than homozygous short-allele probands and heterozygous probands, which made the most commission errors ($F_{2,208} = 5.5$, $P = .04$). These differences were also modulated by the cognitive load: the more difficult the task became, the more severe the differences between the groups ($F_{4,416} = 5.8$, $P < .001$). Interestingly, heterozygous probands made the most errors, repeating the heterosis effect seen in our earlier study (Reif *et al.* 2006a). Also, in another sample of healthy controls, we carried out a combined Stop Signal/Go-NoGo task as this provides insight into several aspects of impulsivity. We demonstrated that *NOS1* Ex1f-VNTR LL (e.g. non-impulsive) carriers displayed increased activity of the dorsolateral prefrontal cortex during NoGo trials and, as predicted, activation in the inferior frontal cortex during successful inhibition in the Stop Signal task, while no significant activation was found in the homozygous short-allele group (Kopf *et al.* 2012). This confirms an influence of *NOS1* ex1f-VNTR on impulsivity; impairment of prefrontal control with consecutive failure of inhibitory processes might underlie the genetic association.

Major depression

As compared with the above phenotypes, much less has been published on a possible connection between genetic variants in *NOS1* and depression and anxiety despite plenty of preclinical data (as reviewed in Dhir & Kulkarni 2011; Doucet *et al.* 2012) and an early promising linkage study (Abkevich *et al.* 2003). Since then, one study on bipolar disorder (Buttenschon *et al.* 2004) and two small Asian studies on major depression argued against a contribution of the *NOS1* gene to affective disorders (Okumura *et al.* 2010; Yu *et al.* 2003), while another dataset (Galecki *et al.* 2011; Sullivan *et al.* 2009) was in favor of an association. A GWAS on major depression also provided evidence for an association of *NOS1* with the disease; however, the authors note that the findings might be false-positive due to the sheer size of the *NOS1* gene (Sullivan *et al.* 2009), so that the finding was not investigated any further. Another GWAS provided evidence for an association of *NOS1* with 'psychological distress' (Luciano *et al.* 2012) and finally, we demonstrated that short alleles of *NOS1* ex1f-VNTR (see above) went along with higher neuroticism and anxiety in a gene × environment interaction manner (Kurrikoff *et al.* 2012). Short-allele carriers had higher neuroticism and anxiety in general, but in the face of environmental adversity, risk allele carriers displayed even higher scores of neuroticism, anxiety and depressiveness. This study is corroborated by a recent report on >1200 individuals (Sarginson *et al.* 2014); here, environmental adversity in the form of financial hardship interacted with *NOS1* genotypes – especially in the regulatory region – on depression scores. *NOS1* therefore seems to increase the risk toward depression only in interaction with adverse environmental

conditions, which should be followed up in further studies.

On a related matter, it was shown in a sample of suicide attempters/completers, mostly consisting of patients suffering from affective disorders, that *NOS1* SNPs are associated with suicidal behavior (Rujescu *et al.* 2007). In further exploratory analyses, the authors demonstrated *NOS1* to be associated with anger and aggression traits; a trend association of *NOS1* with affective disorders in this sample was described as well. We could again replicate the association of short *NOS1* ex1f-VNTR alleles with suicidal behavior in an independent sample mainly consisting of schizophrenic patients (76% of $n = 438$ patients, 142 of which had a history for attempted suicide; $P = 0.04$; in preparation).

Loudness-dependent auditory potentials (LDAEP) are thought to mirror central serotonergic activity and were shown to be altered in a wide range of psychiatric conditions, such a depression, alcohol dependence and schizophrenia. Kawohl *et al.* (2008) correlated *NOS1* SNPs to LDAEP and demonstrated that *NOS1* ex1c-SNP is associated with lower LDAEP. As low LDAEP are considered to reflect higher serotonergic activity, these findings further highlight the tight interactions between the serotonergic and the nitrinergic systems; e.g. it might be speculated that lower *NOS1* expression due to the *NOS1* ex1c-SNP A-allele results in subsequently altered cell surface expression of the 5-HTT (Chanrion *et al.* 2007) thereby decreasing 5-HT uptake. Further studies on the complex interplay between NO and 5-HT in humans thus are necessary, e.g. by analyzing 5-HTT PET ligand binding in correlation to *NOS1* genotype.

Other disorders

An addition to the disorders discussed above, preclinical studies indicate an association of nitrinergic signaling with several other psychiatric conditions, including ASD, anxiety disorders and OCD. However, to date evidence from human studies indicating an involvement of nitrinergic signaling in these disorders is sparse.

Several studies showed elevated levels of NO (nitrite) in the blood (Lakshmi Priya & Geetha 2011), plasma (Essa *et al.* 2012; Sweeten *et al.* 2004; Tostes *et al.* 2012; Zoroglu *et al.* 2003) or red blood cells (Sogut *et al.* 2003) of children with autism. Elevated plasma levels of NO were shown to correlate positively with the amount of interferon gamma, suggesting that NO production in ASD patients is dependent on interferon gamma activity (Sweeten *et al.* 2004; Tostes *et al.* 2012). Only very few genetic studies, however, found an involvement of the nitrinergic system in ASD. A pathway-based outlier analysis on blood transcriptome levels found a significant over-representation of outliers in the nitric oxide signaling pathway (Campbell *et al.* 2013). A relatively small (151 patients) family-based association study found nominal association of two SNPs in the *NOS1* gene with ASD (Kim *et al.* 2009), while another study found one rare mutation in the coding region of the *NOS1AP* gene in three brothers, two of those affected with ASD (the other with social phobia). This mutation was in proximity to the NOS1AP binding motif and was suggested to affect stability of the protein in this region. However, the investigators were not able to find this variation in an extended sample of patients and also failed to detect any significant SNPs in this gene (Delorme *et al.* 2010). Finally, several studies indicated that treatment with tetrahydrobiopterin (H_4Bip) might be beneficial to treat ASD symptoms (Frye *et al.* 2010). H_4Bip is a cofactor of NOSs and is required for NO synthesis, but is also involved in monoamine synthesis.

Regarding anxiety disorders, one study investigated serum nitrite and nitrate levels in patients with panic disorder. When measured in the morning (after overnight fasting), nitrite levels were significantly increased in patients with panic disorder, while nitrate levels only showed a trend toward a significant increase. Interestingly, when measured in the afternoon (2 h after lunch), nitrite and nitrate levels were comparable between patients with panic disorder and control subjects, suggesting a diurnal involvement (Kaya *et al.* 2004). Another study found slightly, but not significantly, elevated levels of serum NO (nitrite) in patients with panic disorder, which were significantly reduced after eight weeks of treatment with selective serotonin reuptake inhibitors (Herken *et al.* 2006). In contrast, in two different studies no alterations in NOS enzymatic activity in platelets were detected in patients with panic disorder (Das *et al.* 1995; Marcourakis *et al.* 2002). Genetic evidence for an involvement of NO signaling is very sparse. In a cross-disorder study we found that short allele carriers of a *NOS1* ex1f-VNTR showed higher anxiety levels than carriers of the long alleles (Kurrikoff *et al.* 2012). The only other genetic study did not find an association of SNPs in the *NOS1* and *NOS3* genes with anxiety in elderly (~79–81 years) patients (Luciano *et al.* 2010).

To date only two human studies provided evidence for an involvement of the NO pathway in OCD. The first study found a significant increase on NO (nitrite and nitrate) in OCD patients vs. controls. Importantly, NO levels were significantly and positively correlated with OCD severity (Atmaca *et al.* 2005). The other study found a rare mutation in the *NOS1AP* gene within the coding region for the phosphotyrosin-binding domain, in two siblings with OCD (Delorme *et al.* 2010). However, since these are the only human studies regarding the NO system in OCD, its involvement in this disorder is still uncertain and requires further validation.

NO-related biomarkers

In general, studies pertinent to detect abnormalities of the NO pathway *in vivo* have the disadvantages of small sample sizes, hardly any replication studies and the transient nature of their candidate molecule: as NO is rapidly metabolized, it cannot be measured directly which hampers its precise determination. Furthermore, most of the studies aim to measure NO metabolites in blood specimens, and whether or not this parallels changes in the CNS is highly controversial. Given these limitations, most of the published studies provided converging, although preliminary, evidence for a disturbance of NO metabolism in schizophrenia and bipolar disorder.

Supporting the above studies on decreased NOS activity in post-mortem brain from schizophrenic patients, significantly lowered NO metabolites (nitrite and nitrate) were found in the

CSF of schizophrenic patients (Ramirez *et al.* 2004) in a pilot study on 10 patients. Correspondingly, it was shown more than 25 years ago that cyclic GMP, formed by the NO receptor enzyme sGC, is decreased in the CSF of schizophrenics (Gattaz *et al.* 1983). In contrast to these *in vivo* measurements, which assess rather global NO formation, higher levels of NO metabolites have been found post-mortem in the caudate nucleus of 18 schizophrenic patients (Yao *et al.* 2004) arguing that the production of NO is increased in this brain area, but probably decreased in others. Given the small number of subjects in each of these studies, these findings have to be considered preliminary. Thus, firm conclusions are not yet justified.

Quite a number of studies have investigated NO metabolites in blood samples with very mixed results. Das *et al.* (1995) demonstrated increased NOS activity in platelets of schizophrenic patients, which mainly express NOS-III and, to a lower level, NOS-II (Wallerath *et al.* 1997). In contrast, other groups investigated plasma samples and found an increase in nitrate in 21 (Atmaca *et al.* 2007), 20 (Taneli *et al.* 2004) and 46 (Li *et al.* 2006; however, nitrite levels in this study were two magnitudes lower than usual) drug-naïve schizophrenic patients. While olanzapine treatment appeared to normalize nitrite levels (Atmaca *et al.* 2007), mixed antipsychotic treatment mainly consisting of risperidone (75% of patients) had no significant effect whatsoever (Taneli *et al.* 2004). Akyol and colleagues repetitively demonstrated elevated NO metabolite levels in the plasma ($n = 82$, 59 males thereof, Zoroglu *et al.* 2002; $n = 66$, entirely male but overlapping with other studies of the same group, Yilmaz *et al.* 2007; $n = 46$, 36 males thereof; Yanik *et al.* 2003) and in red blood cells ($n = 75$; Herken *et al.* 2001) of medicated schizophrenic patients. Erythrocytes do not possess NOSs, but are rather considered as NO vehicles delivering NO to the periphery, as hemoglobin scavenges NO formed by endothelial NOS-III and, interestingly, circulating nitrite (Grubina *et al.* 2007). In contrast, polymorphonuclear leukocytes (PMN) mainly express NOS-I (Wallerath *et al.* 1997). The most comprehensive study thus far examined nitrite levels in PMNs, platelets, as well as in the plasma in 62 un-medicated, schizophrenic patients. A 68% decrease of PMN nitrite was demonstrated, but platelet and plasma nitrite concentrations were not significantly altered (Srivastava *et al.* 2001; however, it has to be said that our re-analysis of the data yielded a highly significant *P*-value, with 25% higher plasma nitrite levels in patients). In line with this, lower NO metabolite levels in the plasma (Das *et al.* 1996; Suzuki *et al.* 2003) have also been demonstrated in schizophrenic patients, although these studies were very small – for instance, the study by Das et al. relied on only 13 drug-naive patients, while three sulpiride-treated patients had normal nitrite levels. The study by Suzuki and colleagues distinguished between deficit ($n = 11$) and non-deficit ($n = 14$) forms of un-medicated schizophrenia; lower levels of nitrate were found in deficit schizophrenia as compared to non-deficit schizophrenia, while no significant difference was found between either conditions and controls.

Comparison of the studies is hampered by the use of different biological specimens (PMN, platelet, RBC, plasma), different methods (Griess reaction, HPLC), technically flawed data presentation, male/female ratio, medication status of the patients, the inherent heterogeneity of schizophrenia and small sample sizes [all studies combined, $n = 345$ cases vs. 266 controls (including Yilmaz *et al.* 2007). If anything can be concluded at all, antipsychotic treatment seems to cause an increase in plasma nitrite/nitrate levels, although one study on olanzapine found just the opposite (Atmaca *et al.* 2007). Furthermore, plasma levels of nitrite seem to be increased in schizophrenia (Fig. 4), which becomes less clear when studies on medicated patients are excluded: by conducting a meta-analysis on all studies (hitherto unpublished data), assuming a random-effects model, the pooled effect size is 0.36 (95% CI −0.47 to 1.19; i.e. small to medium effect of diagnosis on nitrite levels); when only drug-naïve patients are considered, the pooled effect size drops to 0.16 (95% CI −1.29 to 1.61). Computing fixed-effects models yields better pooled effect sizes (0.85 and 1.33, respectively), however are not appropriate given the heterogeneity of the studies (I^2 94% and 96%, respectively). Finally, NOS activity in PMNs might be decreased, which might reflect 'real' genetic underpinnings, however, lacks replication thus far. As PMNs do not express *NOS1* exon 1c or exon 1f (D. Saur, personal communication), it is unclear whether or not the above-mentioned polymorphisms in the promoter regions of these alternative exons have a role here.

In bipolar disorder, elevated nitrate levels were found in studies examining 43 (Yanik *et al.* 2004), or 44 (Savas *et al.* 2002) patients suffering from bipolar-1 disorder, although it appears that both studies present data on the same patient sample. This finding was replicated (Selek *et al.* 2008, 30 bipolar-depression patients; Gergerlioglu *et al.* 2007, 29 manic patients) and was also true in the euthymic phase of bipolar disorder (Savas *et al.* 2006; $n = 27$ patients). More specifically, longitudinal examinations argued that treatment normalizes these elevated NO levels in bipolar depression (Selek *et al.* 2008), but not mania (Gergerlioglu *et al.* 2007). One caveat to bear in mind is that these studies on NO metabolites originate from one single site and partially overlapping samples are reported (we abstained from meta-analyzing these studies; however, another meta-analysis did so and indeed reported significant increase of NO_x in bipolar disorder (Brown *et al.* 2014), which is not stated clearly in every case. Thus, replication is paramount in the appraisal of these findings. In performing a multi-level analysis of the NO signaling system examining NO_x levels (obtained by using a more refined measurement method), NOS expression and genetic variation in *NOS1* and *NOS3*, we also found an increase of NO_x in bipolar disorder, with manic patients having the highest levels (Kittel-Schneider *et al.* 2014). In line with these studies showing elevated NO levels in bipolar disorder, it was demonstrated that bipolar patients have increased 3-nitrotyrosine levels in peripheral blood across all stages of the disease (Andreazza *et al.* 2009, 2010). Taken together, evidence for increased NO production at least in the periphery in bipolar disorder is more compelling and fits well with the genetic data showing that a haplotype which is protective against bipolar disorder (Reif *et al.* 2006b) presumably leads to decreased NO production.

For studies on both schizophrenia and bipolar disorder, it has to be kept in mind that NO metabolite measurements in plasma were mainly conducted by the Griess method: a

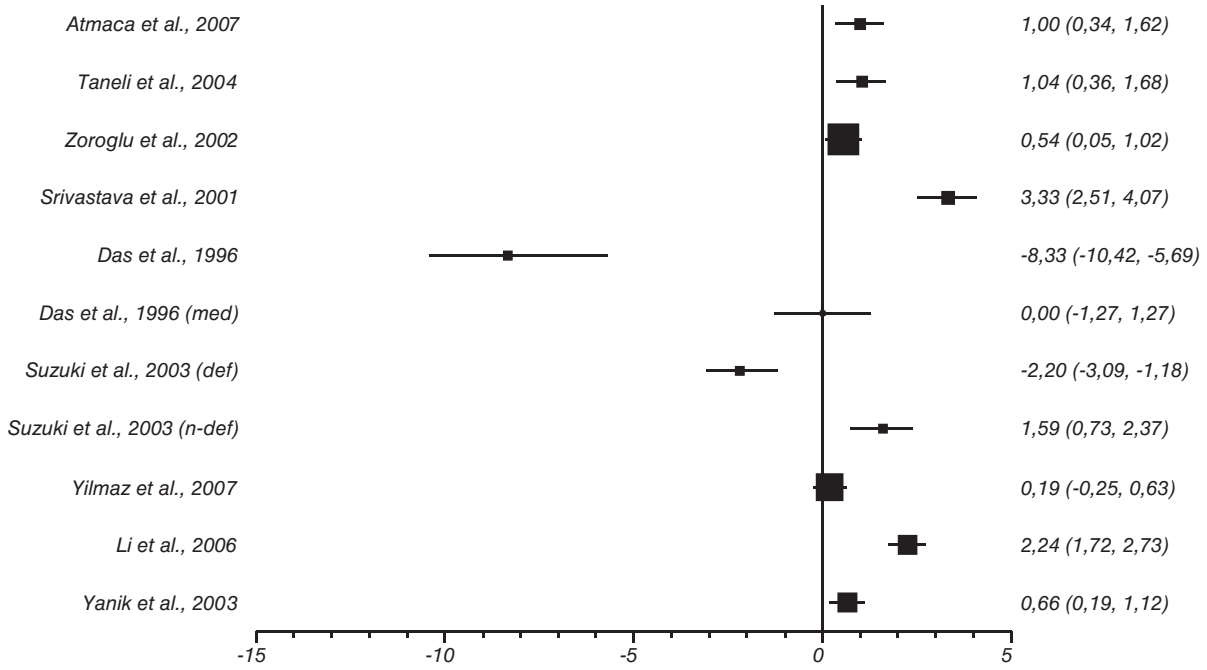

Figure 4: Meta-analysis of NO_x-levels in schizophrenia. Analysis on pooled data from drug-naïve and drug-treated patients resulted in a pooled effect size of 0.36 assuming a random-effects model (95% CI −0.47 to 1.19; i.e.). When restricting analysis to drug-naïve patients only, the pooled effect size drops to 0.16 (95% CI −1.29 to 1.61). The *x*-axis provides effect size along with the respective confidence interval, the size of the square is proportional to the weighting of the study. med, medicated; def, deficit syndrome; n-def, remitting (non-deficit) schizophrenia.

mixture of NO metabolites coming from white blood cell NOS-I, platelet NOS-III, hemoglobin-released NO mainly originating from vasculature NOS-III and genuine plasma nitrate are measured all together. As a plethora of variables impact on each source, and as also parameters like redox state, anti-oxidant enzymes, etc., are of central importance, the underlying reason for plasma nitrite abnormalities in schizophrenia or bipolar disorder is elusive. Finally, the majority of plasma nitrite comes from the periphery, but not the CNS, so that it is even more doubtful that these parameters actually reflect NO neurotransmission in the brain. Rather, they might be considered epiphenomena, whose investigation might still be worthwhile, as, e.g. NOS-III regulation in the CNS and the periphery might be achieved in a similar manner.

Additional observations include increased levels of ADMA (Das *et al.* 1996) – which, however, also takes place in major depression (Selley 2004) –, an endogenous NOS inhibitor, and lowered levels of arginase (Yanik *et al.* 2003), which competes with NOS for substrate, in blood samples from schizophrenic patients. A few further studies determined blood levels of tetrahydrobiopterin, an essential cofactor of NOS, in schizophrenia and bipolar disorder. Richardson and colleagues found a 34% decrease in plasma biopterin in 154 schizophrenic subjects; a genetic analysis in a subset of subjects did not reveal mutations in the coding region of the GTP cyclohydrolase I feedback regulatory protein gene GFRP, which regulates tetrahydrobiopterin synthesis (Richardson *et al.* 2005). This group later expanded their findings to patients with schizoaffective and bipolar disorder, where a decrease in plasma biopterin could be observed as well, however, not as pronounced in the bipolar group (Richardson *et al.* 2007). Another study also demonstrated decreased neopterin levels in euthymic and disordered bipolar patients ($n = 32$, and 12, respectively, vs. 20 controls; Hoekstra *et al.* 2006). In contrast, patients suffering from major depression had normal levels. Finally, a metabolomic study (He *et al.* 2012) demonstrated that L-arginine levels were decreased in both medicated as well as drug-naïve patients suffering from schizophrenia, which was put into perspective by the authors by suggesting that the NO pathway as such is altered in schizophrenia.

Fewer studies have examined NO biomarkers in major depression; as with schizophrenia and bipolar disorder, findings are rather mixed. In another study from the Akyol laboratory, in un-medicated patients, NO metabolite plasma levels were found to be nominally, albeit non-significantly, increased, which however were significantly lowered upon antidepressant treatment (Herken *et al.* 2007). Interestingly, the same was true for panic disorder arguing for specificity of treatment, but not diagnosis (Herken *et al.* 2006). These data were underscored by studies demonstrating increased nitrite/nitrate levels in suicide attempters, irrespective of diagnosis (Kim *et al.* 2006; Lee *et al.* 2006; partially overlapping samples were presented in these papers). A further study employing HPLC detection of nitrite also found elevated

nitrite levels in 17 drug-naïve patients suffering from depression (Suzuki *et al.* 2001). A relatively large, independent examination from Poland again suggested that plasma NO_x levels are significantly increased (almost doubled) in major depression, and that, interestingly, increased NO_x levels coincided with cognitive impairment – especially working memory – and higher depressive symptoms (Talarowska *et al.* 2012). This finding was recently conformed in a Turkish study (Akpinar *et al.* 2013) on 50 patients suffering from depression; again, depression went along with higher NO_x levels which interestingly correlated with psychomotor retardation. The connection of increased peripheral NO_x levels with frontal, executive functions in both studies is unclear but definitely warrants further investigations.

In contrast to the findings in plasma specimens, which reflect rather various sources of NO (as outlined above), nitrite content of polymorphonuclear leukocytes was reduced by 73% in 66 depressive patients (Srivastava *et al.* 2002). In line with this, but also contrasting the above studies, significantly decreased platelet NOS activity (Chrapko *et al.* 2004; Pinto *et al.* 2012) as well as plasma NO_x (measured by chemiluminescence) was found in 15 drug-naïve patients (Chrapko *et al.* 2004), along with normal biopterin concentrations. This was later replicated by the same group in 17 patients suffering from major depression (Chrapko *et al.* 2006) and extended by the finding that paroxetine treatment increased plasma NO_x, although not to the level of normal controls. Corroborating this assumption, paroxetine also led to an increase in nitrite/nitrate plasma concentrations in healthy controls (Lara *et al.* 2003). Finally, decreased NO_x levels were measured in the CSF of depressed patients which was paralleled by reduced NOS-I immunoreactivity in the ACC (Gao *et al.* 2013).

Therefore, no firm conclusions can be made about whether or not NO_x are altered in major depression. The data reviewed above suggest that in major depression, blood NO_x levels are increased, while NO production in platelets and the CNS seems to be decreased. Findings also converge to the notion that SSRI treatment increases NO metabolites. The underlying mechanism remains elusive; however, as platelets co-express NOS-II/III and the serotonin transporter, an effect of SSRIs on NO_x levels in blood, however, seems mechanistically conceivable. Therefore the finding of plasma NO_x increase in depression might represent a treatment effect mainly reflecting increased NO production in platelets, although it seems equally possible that increased NO_x plasma levels are a general feature of mood disorders.

Summary and conclusions

Taken together, preclinical models that are reviewed elsewhere and human genetic data from our and other laboratories, as outlined above, lead to the following major hypothesis regarding *NOS1*'s contribution to disease:

1 Genetic variation and dysregulation in prefrontal *NOS1* contribute to schizophrenia and cognitive deficits.
2 Downregulation of striatal *NOS1* goes along with a variety of impulsive phenotypes.

These considerations assume that tightly regulated NO levels are crucial for exerting differential effects on various behavioral domains and hence mental disorders. No simple unidirectional relationship between genetic variance in the *NOS1* gene and psychiatric diseases can be made. Rather, one has to consider regional and cell-type-specific dysfunction of the nitrinergic system that contributes to disturbances of specific neurocircuits. This in turn underlies neuropsychological endophenotypes associated with disease. Therefore, it is critical to uncover these pathophysiological mechanisms from molecule to behavior in order to understand the contribution of the NO system to normal and disordered function of the CNS. Further adding to this, there are pilot trials on modulating the NO system in schizophrenia (Hallak *et al.* 2013; Wass *et al.* 2011) and bipolar disorder. Probing the NO system, either on the genetic level or on the biomarker level, in this context might serve to predict treatment response ('precision medicine'), although respective studies have yet to be conducted.

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