Shank3 mutation in a mouse model of autism leads to changes in the S-nitroso-proteome and affects key proteins involved in vesicle release and synaptic function

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Abstract
Mutation in the SHANK3 human gene leads to different neuropsychiatric diseases including Autism Spectrum Disorder (ASD), intellectual disabilities and Phelan-McDermid syndrome. Shank3 disruption in mice leads to dysfunction of synaptic transmission, behavior, and development. Protein S-nitrosylation, the nitric oxide (NO•)-mediated posttranslational modification (PTM) of cysteine thiols (SNO), modulates the activity of proteins that regulate key signaling pathways. We tested the hypothesis that Shank3 mutation would generate downstream effects on PTM of critical proteins that lead to modification of synaptic functions. SNO-proteins in two ASD-related brain regions, cortex and striatum of young and adult InsG3680(+/+) mice (a human mutation-based Shank3 mouse model), were identified by an innovative mass spectrometric method, SNOTRAP. We found changes of the SNO-proteome in the mutant compared to WT in both ages. Pathway analysis showed enrichment of processes affected in ASD. SNO-Calcineurin in mutant led to a significant increase of phosphorylated Synapsin1 and CREB, which affect synaptic vesicle mobilization and gene transcription, respectively. A significant increase of 3-nitrotyrosine was found in the cortical regions of the adult mutant, signaling both oxidative and nitrosative stress. Neuronal NO• Synthase (nNOS) was examined for levels and localization in neurons and no significant difference was found in WT vs. mutant. S-nitrosoglutathione concentrations were higher in mutant mice compared to WT. This is the first study on NO•-related molecular changes and SNO-signaling in the brain of an ASD mouse model that allows the characterization and identification of key proteins, cellular pathways, and neurobiological mechanisms that might be affected in ASD.

Introduction
Autism spectrum disorder (ASD) is a highly heritable neurodevelopmental syndrome, caused by genetic modifications [1–3] as well as non-genetic factors [4, 5]. These alterations may lead to improper development of synapses or brain circuits [6], resulting in impaired social behavior [6], along with stereotyped, repetitive behavior with narrowly restricted interests [7]. Human genetic studies have identified deletions/mutations of the SHANK3 gene as the cause of Phelan-McDermid Syndrome (PMS), a neurodevelopmental disorder with >50% of the patients having a diagnosis of ASD [8]. Recent genetic screens have identified many mutations/variants of the SHANK3 gene in ASD patients outside of a diagnosis of PMS suggesting that SHANK3 mutations also contribute to non-syndromic ASDs [9–12]. Shank3 is a member of the Shank family of proteins (Shank1–3) [13–16]. Shank interacts with many postsynaptic density (PSD) proteins. Most notably, Shank
binds to SAPAP, which in turn binds to PSD95 to form the PSD95/SAPAP/Shank postsynaptic complex [17]. Altogether, these three groups of multi-domain proteins are proposed to form a key scaffold for assembling the macromolecular postsynaptic complex at glutamatergic synapses. This complex has been shown to play important roles in targeting, anchoring, and dynamically regulating synaptic localization of neurotransmitter receptors and signaling molecules [18, 19]. Shank is also connected to the metabotropic glutamate receptor (mGluR) pathway through its binding to Homer [20]. Together, these data strongly suggest that Shank proteins play key roles in synaptic development and function [21, 22]. Shank3 is highly enriched at corticostriatal glutamatergic synapses [16, 23], part of the neural circuits strongly implicated as dysfunctional in ASDs [24–27]. In the cortico-striatal-thalamo-cortical circuitry, information from various cortical areas converges on the striatum and the processed information returns to the cortex through the thalamic relay to guide motor and behavioral decisions. Thus, diverse synaptic defects in different parts of the circuitry will all end up affecting cortical activity that could be a converging mechanism for repetitive behaviors [28] with different genetic causes and in different disorders. Previous studies on mouse models showed that disruption of Shank3 resulted in both structural and functional changes in corticostriatal synapses [23, 27, 29–31], while oppositely, adult restoration of Shank3 expression rescued selectively the behavioral and physiological deficits in mice [32]. Thus, the Shank3 gene provides us with a unique opportunity to dissect cellular and molecular mechanisms of ASD-relevant behavioral abnormality [33]. It is not clear how such diverse genetic mutations and consequent synaptic dysfunctions converge to cause core ASD symptoms such as repetitive behaviors and social communication deficits. A plausible hypothesis is that ASD symptoms are the result of dysfunction of core behavior-relevant circuitry that could be caused by various forms of synaptic dysfunction [6, 34]. Such a dysfunction could be due to improper protein-protein interactions and altered molecular pathways. For instance, a mutation can lead to critical modifications in protein structure, which may affect its ability to interact with other proteins and its functionality. To study the proteomic consequences of Shank3 mutation in the cortico-striatal circuitry, we used the recently published human mutation-based mouse model for ASD (InsG3680(+/+)), in which a guanine nucleotide was inserted at cDNA position 3680 of Shank3 gene, leading to a frameshift that results in a premature stop codon [29]. These mice were shown to have striatal and cortico-striatal synaptic transmission and social behavioral defects, along with intense overgrooming repetitive behavior [29]. Understanding the nature of this circuitry imbalance and its consequences on circuitry output relevant to ASD behaviors is a critical step for defining converging neurobiological mechanisms and for developing new strategies for effective treatment.

Nitric oxide (NO) is a signaling molecule in the central and peripheral nervous system and is one of the most important messenger molecules [35, 36]. NO* produced in the brain from L-arginine by three nitric oxide synthase isoforms (NOS1, NOS2, NOS3) [37]. NOS1 (also known as neuronal NOS - nNOS) is primarily expressed and regulated in neurons, NOS2 in microglia, astrocytes, and some neurons, and NOS3 in blood capillaries [38]. At low concentrations NO* acts as a signaling molecule, and at higher concentrations may cause modified phenotypes and cell death [39]. However, under some conditions NO* has neuroprotective properties and may have therapeutic value for brain injury [40]. NO* has a critical role in synaptic transmission in the brain [36] and plays a major role in signaling through activation of GMP cyclase [36], effects on ion transporters [36], and S-nitrosylation (SNO) of peptides and proteins [41, 42]. SNO, like phosphorylation, is a reversible posttranslational modification (PTM) in which a cysteine is converted to a nitrosothiol. SNO regulates the localization and activity of many key enzymes and receptors [39, 43, 44] leading to modulation of many canonical signaling pathways, synaptic plasticity, axonal elongation, movement of proteins to the cell membrane, and protein assembly [39, 43].

NO* also plays a major role in inflammation-induced oxidative/nitrosative stress, leading to damage to biological molecules, including protein tyrosine nitration (formation of 3-nitrotyrosine) [45–47]. S-nitrosoglutathione (GSNO) forms at diffusion-controlled rates in a radical recombination between NO* and GSH thiyl radicals (RS•), and can then transnitrosate other thiols on peptides and proteins [42, 48, 49].

In the brain, numerous examples of neuropathology exist that involve SNO [50–52] such as Alzheimer’s [39, 53, 54], Parkinson’s [55] and Huntington’s disease [39, 54]. In a mouse model of Alzheimer’s disease [53], NO* output greatly increases at the locus of the pathology, leading to changes of the SNO-proteome and consequently all of the functions mentioned above. However, previous literature has demonstrated that de-nitrosylation could lead to neuronal apoptosis [56] and dysregulated SNO may contribute to a range of human pathologies [57]. We now know that the status and composition of the SNO-proteome is regulated by a combination of transnitrosylating peptides and proteins [42, 58] balanced by de-nitrosylating peptides and proteins [57], but the details of this regulation are unknown. An example of a protein that can nitrosylate or de-nitrosylate, depending on its oxidative state is thioredoxin [57, 59].

In the case of ASD, there is currently no prior evidence for the involvement of NO* and S-nitrosylation in the pathology. The literature is dominated by genetic and epigenetic analysis of human brain, and the development of mouse models based upon the human database of autism-associated genes.
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Database for Annotation, Visualization and Integrated Discovery (DAVID) [73] Bioinformatics Resources (version 6.8, https://david.ncifcrf.gov). STRING (version 10.0) was used to analyze the protein-protein interaction of SNO-proteins (http://string-db.org). For more information on DAVID, STRING, and the statistical analysis, see SI.

For materials and reagents, sample preparation of brain tissues for MS, MS analysis, WB and IHC methodology, and mathematical models, see SI.

Results

We studied two regions in the mouse brain, which are of special interest due to their role in ASD pathology, cortex and striatum. Because ASD is a neurodevelopmental disorder, we studied NO\textsuperscript{•} involvement developmentally, using mice at two different ages: 6 week-old and 4 month-old. In total, 8 different groups were analyzed: 6 weeks-cortex-WT (6w-cor-WT), 6 weeks-cortex-KO (6w-cor-KO), 6 weeks-striatum-WT (6w-str-WT), 6 weeks-striatum-KO (6w-str-KO), 4 months-cortex-WT (4m-cor-WT), 4 months-cortex-KO (4m-cor-KO), 4 months-striatum-WT (4m-str-WT), and 4 months-striatum-KO (4m-str-KO). See Fig. 1 for a schematic of the study strategy.

**Shank3 mutation leads to changes in the SNO-proteome of 6w and 4 m InsG3680(+/+) mice**

The SNO-proteomic analysis revealed differences in SNO-proteins between the KO and WT in both ages (6w and 4 m) and regions (cortex and striatum). For all proteins identified in the eight different groups, see Supplementary Table 1. Because tandem MS identifies a subset of sample proteins
stochastically, we took three approaches to confirm that the proteins identified in WT and KO mice are not likely to be different subsets of the same overall pool. First, we built simple mathematical models describing the approach of our technical and biological replicates towards an asymptote under the assumption that each replicate within a condition stochastically sampled a fraction of an identical pool (Supplementary Figure 1). Second, for 10 proteins identified in the KO but not WT mice, we manually searched the WT MS data for evidence that the proteins were present at the correct chromatographic retention time and mass, but not fragmented for the second MS stage. For 7/10 proteins, we found no such evidence. For the remaining 3, an MS1 peak plausibly associated with the protein was found, but the protein was in insufficient quantity to be selected for fragmentation. Finally, WBs were performed in SNOTRAP eluates for two key proteins detected only in KO samples, calcineurin (CN) and syntaxin 1a (Stx1a). The proteins were seen only in KO eluates (Fig. 3a, b). Taken together, these results indicate that it is unlikely that the set of SNO-proteins is unaltered between the WT and KO conditions.

Proteins associated with processes and pathways known to be affected in ASD are enriched in KO SNOTRAP samples

To gain a systems-level insight into SNO-proteins’ functionalities, and to test whether enriched processes are related to ASD, we performed GO-based protein enrichment analysis of the cortex and striatum for both 6w-old and 4m-old WT and KO mice. Three categories were analyzed in DAVID: CC, BP, and KEGG pathways. We identified processes and pathways in each tissue type with statistically significant enrichment of S-nitrosylated proteins vs. a background of the total proteome, and then focused on the set of these processes and pathways that differentiated healthy from Shank3 mutant mice. For example, in adult KO cortex samples, 9/121 proteins identified by SNO-trapping were annotated with the KEGG pathway “synaptic vesicle cycle”, including Stx1a, synaptotagmin 1, N-ethylmaleimide sensitive fusion protein (Nsf), and others. This represents more than a 9-fold enrichment over the proteomic background, with a nominal p-value of $4.5 \times 10^{-6}$ and a Benjamini-Hochberg corrected FDR of $2.7 \times 10^{-4}$. By contrast, proteins associated with synaptic vesicle formation are not significantly enriched in the adult WT cortex sample.

6w-cor-KO showed a significant enrichment of different GO terms and KEGG pathways that in part are associated with ASD such as nervous system development (Fig. 2a). None of these was found in 6w-cor-WT, emphasizing the association between SNO and InsG3680(+/+) (see Supplementary Table 2). Testing the 4m-cor-KO group has also revealed enriched pathways such as synaptic vesicle cycle (detailed above) and oxidative phosphorylation (Fig. 2c, Supplementary Table 2). None of these processes was found in 4m-cor-WT (Supplementary Table 2).

For detailed lists of the CC, BP and KEGG annotations enriched in all 8 tested groups, see Supplementary Table 2. Further, Supplementary Table 3 lists 49 SNO-proteins (in 4m-cor-KO) in 5 important pathways and functions (from GO and KEGG) that are found in KO, not in WT, and may reasonably be linked to pathology. Also shown in Supplementary Table 3 are the results for 6w-cor-KO.

SNO-protein interactome analysis reveals synaptic proteins clusters in the cortex of InsG3680(+/+) mice

To test for functional and physical interactions among SNOed proteins in the InsG3680(+/+) mice, we used STRING to analyze protein-protein interactions. Clusters of SNO-proteins represent a group of proteins with a common function that are joined because of their proximity or physical interactions. In other words, it may be a concerted process. For all network clusters of the 8 groups, see Supplementary Figures 2 & 3.

Analysis of cor-KO mice at both ages showed networks of SNO-proteins related to processes involved in ASD. Proteins that were SNOed and are functionally related to synaptic vesicle-cycle and neurotransmission (Ppp3ca, Stx1a, VAMP3, and others) and to the glutamatergic synapse (mGluR7, Glud1, Gnao1, and others) were interconnected (See Fig. 2b, d). Proteins that were clustered in 6w-cor-KO are functionally associated with “synaptic vesicle cycle and neurotransmitters release regulation”, “vesicle trafficking and fusion” and also “glutamatergic synapse”, suggesting that S-nitrosylation may be involved in an early phase of InsG3680(+/+) mouse model pathology (Fig. 2b). 4m-cor-KO mice showed very similar results (Fig. 2d). Clusters related to “glutamatergic synapse and glutamate regulation” and “synaptic vesicle cycle and neurotransmission” were observed. Supplementary Figure 4 lists in details lists of proteins in cor-KO groups that were clustered and are functioning in processes known to be correlated with ASD. Regulatory proteins and processes were clustered in the cor-WT groups such as “ATPases” proteins in 6w-cor-WT and “cell cycle” and “transcript regulation” processes in 4m-cor-WT (Supplementary Figure 3).

Shared SNO-proteins of 6w-old and 4m-old mice in the cortex are enriched for processes and interactome clusters known to be affected in ASD

We studied the shared proteins that were SNOed and found in 6w-old and 4m-old mice (See Supplementary Figure 5
and Supplementary Table 1). GO analysis was conducted to decipher the possible impact of the shared proteins of both ages on the cellular systems. The shared proteins of the cor-KO groups showed an evidence of enriched process known to be affected in ASD such as "synaptic vesicle cycle" (Fig. 2e and Supplementary Table 2). None of these processes were enriched in the other comparisons (see Supplementary Figure 5).

Interactomic analysis of the shared proteins of cor-KO proteins showed clusters of SNO-proteins that function in "glutamate regulation" (Got1, Got2, and Gnao1) and "synaptic vesicle cycle" (Stx1a, Ppp3ca, Nsf, and Dnm1). (Fig. 2f).

**Significant increase of 3-nitrotyrosine in the cortical regions of 4m-KO mice**

Ntyr is a product of NO\(^+\) biochemistry participants, particularly peroxynitrite, and proteins become nitrated under conditions of NO\(^+\) overproduction [74]. Therefore, we examined brain regions with potential elevation of NO\(^+\) levels. IHC staining of Ntyr was performed in cortical regions that have functional relevance to the behavioral and physiological deficits in ASD [6]: prefrontal cortex (PFC), motor cortex (MC) and somatosensory cortex (SSC), as well as striatal regions: central, medial, and lateral striatum. The 6 week-old mice showed no significant difference between the KO and the WT groups (Supplementary Figure 6). The 4m-KO mice had increased Ntyr levels in the cortex and the striatum (See Fig. 4a, b, and Supplementary Figures 7,8). Morphometric analysis of the Ntyr level in the cortical regions showed significant increase (p < 0.05) in 4m-KO compared to 4m-WT (Fig. 4c). Further, an increase of Ntyr was also found in the striatal regions of 4m-KO compared to 4m-WT mice (Fig. 4c). Supplementary Figures 6 and 7 show the intensity of Ntyr in each specific cortical and striatal region in both 6w-old and 4m-old mice, respectively. The presence of Ntyr indicates an increased level of nitrosative stress in the cortex and striatum of InsG3680(+/+) mice.

**SNO-calcineurin leads to increase of phosphorylated (P) synapsin1 (ser62, ser67) and P-CREB (ser133) in 4m-cor-KO**

The phosphatase calcineurin (CN, Ppp3ca-catalytic subunit) was SNOed in both ages of cor-KO mice. It was previously shown that nitrosylating compounds inhibit the activity of purified CN and of CN present in cell lysates [75]. To test the effect of S-nitrosylation on CN activity, we quantified
two different known phosphorylated substrates: synapsin1, located in the presynapse [76] and cAMP-response element binding protein (CREB), located mainly in the postsynapse [77].

By WB, we found a significant increase of P-synapsin1 (Ser 62, Ser 67) in 4m-cor-KO compared to 4m-cor-WT (Fig. 3c, e). No significant changes were observed to P-synapsin in 6w KO vs. WT mice (Fig. 3c, d). We also found similar results with P-CREB (Ser 133), a significant increase of P-CREB in 4m-cor-KO but not in the 6w-cor-KO group (Fig. 3f–h). The no difference in the 6w groups may related to the fact that Ppp3cb (a different catalytic subunit in CN) was SNOed in 6w-cor-WT.

**Examination of nNOS levels, nNOS localization, and GSNO levels in both WT and KO groups**

In order to give a mechanistic explanation for the changes in SNO and Ntyr levels between the WT and KO groups, we have tested nNOS levels and its localization. We examined whether nNOS protein expression changed in the WT compared to KO groups in both cortex and striatum tissues of 6w and 4m mice. We found no changes in protein expression between WT and KO groups see Fig. 4d and Supplementary Figure 9.

Further, we tested whether nNOS localization is different between WT and KO. The co-localization staining of...
nNOS and NeuN shown in Fig. 4e, f shows that nNOS is localized mainly in the cell body of the neuron cells in both the WT and KO groups.

To test whether the nNOS activity was altered, we measured S-nitrosoglutathione (GSNO) levels [72] in all WT and KO samples (Fig. 4g) and identified an increase of GSNO levels in the KO compared to the WT. The cortex of both ages showed significant differences in GSNO level, with an increase in both 6w-KO compared to 6w-WT and 4m-KO compared to 4m-WT. An increase was found in the str-KO compared to str-WT in both ages as well. The results point out the possibility of an increase of nNOS activity leading to increase of GSNO concentrations.

**Discussion**

Our study was designed to test the hypothesis that a mutated gene associated with human ASD would generate downstream effects on PTM of critical proteins that could lead to modification of synaptic functions. A significant body of published literature (reviewed in [39]) supports S-nitrosylation (SNO) as a protein PTM that leads to neuronal pathology.

The changes in the SNO-proteome found in the InsG3680(+/-) are a consequence of Shank3 mutation. That SNO-proteins were found exclusively in WT but not in KO, and vice versa shows that both nitrosylation and
de-nitrosylation may be dysregulated in InsG3680(+/+) mice, leading to the small overlap between the groups.

Previous studies examined cellular, electrophysiological, and biochemical defects in the cortico-striatal circuits of the Shank3-KO model [16, 23, 29]. However only a few studies have looked at protein PTMs. One such study recently investigated changes in kinases and phosphatases that lead to alteration of the protein phosphorylation landscape [78].

In order to identify biology influencing or influenced by S-nitrosylation in the context of the ASD phenotype, we looked for processes and pathways significantly enriched among the proteins differentially nitrosylated in healthy and disease-model mice. GO and KEGG analysis revealed that deficiency of Shank3 leads to SNO-modulated proteins in the cortex that in part are functioning in ASD-related processes (Fig. 2). Earlier work has reported that Shank3-KO mice and other ASD models have a deficiency in glutamatergic synaptic neurotransmission, which include deficits in exocytosis, endocytosis and also imbalance of calcium regulation [16, 23, 29, 79, 80]. GO analysis of the shared synaptic proteins between 6w-cor-KO and 4m-cor-KO showed enrichment of the synaptic vesicle cycle and points out the possibility that these SNO-proteins may be involved in Shank3 pathology from young to adult age (Fig. 2e).

The network clusters in Supplementary Figures 2 and 3 analyzed by STRING demonstrate that age and regional locations lead to different changes in function for different classes of neurons in both cortex and striatum.

One of the shared SNO-proteins in the cortex of KO mice in both ages is CN, which is the only Protein Phosphatase (PP) in the brain activated by Ca^{2+} and a major regulator of key proteins essential for synaptic transmission and neuronal excitability [81, 82]. It has been shown that dysregulation of CN leads to different neurological disorders [83–85]. It was also previously shown that nitrosylating compounds inhibit the activity of purified CN and of CN present in cell lysates [75].

To confirm that SNO inhibits the phosphatase activity of CN, we have tested two CN substrates, synapsin1 and CREB. It is important to note that CN is the major Ser/Thr phosphatase that is a sensitive target for inhibition by NO comparing to PP1 and PP2, meaning that the changes in the de-phosphorylation state of its substrates affected by nitrosative stress would be caused mainly by CN and not other PPs [75]. Further, synapsin1 dephosphorylation (P-Ser62, P-Ser 67) sites are highly specific to CN and not to PP1 or PP2 [86]. In the pre-synapse, CN is abundant and regulates key proteins involved in neurotransmission [82]. Synapsin1 is one of the key proteins controlling the vesicles for exocytosis by modulating its binding and trafficking as well. In the de-phosphorylated state, synapsin1 tethers synaptic vesicles and anchors them to actin-based cytoskeleton that maintains the vesicles in the reserve pool (RP) [87], but once phosphorylated, synapsin1 is detached from vesicles and leads to its mobilization from RP to a readily releasable pool (RRP) [76, 88] (See Fig. 5). The increased P-synapsin1 in 4m-cor-KO (Fig. 3c, e) supports the previous evidence that CN is inactivated by SNO and may lead to an increase of vesicle mobilization. To further validate CN inhibition by SNO, we have tested CREB, an important transcription factor that is also regulated by CN in neurons [89, 90]. CREB dephosphorylation is modulated by CN [91]. Our data showed consistency with the synapsin1 findings with an increase of P-CREB (Ser133) in 4m-cor-KO (Fig. 3f, h).

P-CREB (Ser133) is involved in brain development and leads to an increase of gene expression by recruiting transcriptional co-activators [77] (Fig. 5). Our data are also consistent with previous studies that found an increase of P-CREB (Ser133) in an ASD murine model [92] and Fragile X mouse model [93]. Other work also has shown increase of different transcription factors in ASD [94, 95]. Here we provide a molecular interpretation of the elevated P-CREB levels.

Another protein that was SNOed in the cortex of both ages is Stx1a which is a central coordinator for the vesicle exocytosis machinery [96]. Stx1a interacts with synaptobrevin in the vesicle with SNAP25 and forms the SNARE complex [97] (Fig. 5). Munc18 also binds to Stx1a [96] in its closed conformation, inhibits Stx1a to bind to other SNAREs and execute vesicle fusion [98] (See Fig. 5). It was shown previously that incubation of proteins with nitrosative agents leads to increased formation of SNO complex and inhibits binding of Munc18 to Stx1a [99]. Furthermore, S-nitrosylation of Stx1a was shown to act as a molecular switch inhibiting Munc18 binding to Stx1a [98]. This in turn leads to SNARE complex preclusion, facilitating Stx1a binding to the SNARE, and increasing the vesicle fusion machinery (Fig. 5) [98]. Towards this end, SNO-Stx1a in the cor-KO mice may have a critical role in facilitating vesicle fusion in Shank3-KO mice. Another mechanism that may lead to facilitating vesicle fusion is through mGluR7, which is highly localized in the pre-synapse [100] and SNOed in 4m-cor-KO. It is also proposed that in a resting state, mGluR7 binds Gβγ subunits; this binding inhibits the subunits from suppressing the voltage sensitive calcium channels leading to increase of Ca^{2+} influx in the pre-synapse [100] that could facilitate vesicle docking and fusion. We suggest that SNO of mGluR7 inhibits the receptor propagation by binding to the free cysteine [101] leading to the rest-state mode, which lead to increase of Ca^{2+} influx in the pre-synapse and facilitate vesicle fusion (see Fig. 5).

Our data, suggesting the potential increase of synaptic vesicle release and cortical activity, is consistent with previous data showing that deletion of Shank3 leads to an
increase in cortical activity [102]. Further, the increase in P-CREB levels in the cortex supports our suggestion of increased cortical activity. Note that young or adult mice were not tested in the forementioned study, but only early postnatal mice [102].

Further, we have tested Ntyr levels (Fig. 4a–c) in both age groups and found a significant global increase of Ntyr in the 4m-KO mice in the cortical regions. The increased Ntyr formation could potentially be cytotoxic, leading to neuronal loss in certain neuron types [103, 104]. Generally, Ntyr forms from the radical recombination of NO• and O2•− to form peroxynitrite and is the fastest reaction of NO• in cells. Peroxynitrite reacts with tyrosine to form Ntyr and it is probable that the mutant cells develop a form of metabolic stress that increases the production of O2•− by mitochondria, and with increased NO• leads to increased Ntyr.

To understand the mechanism behind the large changes in the SNO-proteome and Ntyr we measured nNOS levels and localization and found no change between the groups. It is now understood that one of the mechanisms for S-nitrosylation is transnitrosation by GSNO [42, 58]. Other nitrosylating agents are also responsible for S-nitrosylation such iNOS-S100A8/A9 [105], Thioredoxin, GAPDH, and others [58]. The elevated GSNO levels in the KO groups indicate the possibility that nNOS activity is increased, possibly through release of Ca2+ from perinuclear proteins (based on unpublished experiments with embryonic neurons in vitro). This would lead to changes in NO•, SNO-proteins,
and Ntyr levels in the KO group consistent with our observations.

In the case of protein phosphorylation there are kinases and phosphatases, and their balance determines cell function. We like to think of S-nitrosylation in a similar fashion with nitrosylases and de-nitrosylases. We now know about multiple SNO-peptides and proteins that drive transnitrosation and something about their specificity, but little about de-nitrosylases. We also know very little about the effect of SNO on protein function unless we have made that protein a specific target for further investigation.

In this study we did not account for de-nitrosylation that acts through different de-nitrosylases such GSNO reductase which regulates GSNO levels [106, 107], thioredoxin reductase, Xanthine oxidase, and others, although it is obvious that it is an important factor [58]. Genetic deletion of GSNO reductase in liver leads to a substantial increase in GSNO and subsequent liver cancer [108]. This study focused on the idea of looking at the entire SNO-proteome in WT and in a single genetic alteration and then looking at the biological effect using GO, pathway, and connectivity analysis to understand some of the downstream effects of the mutation.

Although our mouse model represents a mutation in a gene encoding a postsynaptic protein, substantial presynaptic modifications were characterized in our current study. These transsynaptic changes can be the result of the inability of Shank3 to signal properly to the presynaptic site [109].

A previous in vitro study found a robust changes in synaptic transmission, without alterations in presynaptic protein levels [109]. This suggests that posttranslational modifications, such S-nitrosylation may affect presynaptic proteins’ function, such as Stx1a, CN, VAMP and others that may alter neurotransmitter release.

To summarize, our findings show for the first time the involvement of NO in an ASD mouse model (InsG3680 (+/+)). We have shown that S-nitrosylation is shifted in KO mice compared to WT. GO analysis demonstrated that SNO in KO mice affects processes and pathways known to be involved in ASD. SNO of critical proteins was identified in both ages of KO mice suggesting that NO, is involved in the pathology from a young age. We have shown that SNO-CN led to a significant increase in P-synapsin1 and P-CREB, which affect vesicle mobilization and gene transcription, respectively. We also suggest that SNO-Stx1a and SNO-mGluR7, which were identified in our study, regulate vesicle docking and calcium influx, respectively. Lastly, the nNOS and GSNO results point out the possibility of an increase of nNOS activity in the mutant mice.

Finally, while in this study we characterized a mono- genic mouse model, there are hundreds of genes associated with ASD. The conceptual research approach we have taken here, of examining a specific modification of proteins allows a systems-level characterization and identification of key proteins, cellular pathways and neurobiological mechanisms that might be affected in ASD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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