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Nitric oxide promotes Arc turnover via protein S-Nitrosylation

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Abstract

The activity-dependent turnover of the immediate-early gene product Arc contributes to synaptic plasticity by regulating AMPA receptor trafficking. Although Arc transcription and translation have been extensively studied, the mechanisms governing its rapid degradation remain incompletely understood. Here, using quantitative proteomics together with cultured neurons and an *in vivo* fear-conditioning paradigm,

we identify neuronal nitric oxide synthase (nNOS)-derived nitric oxide (NO) as a regulator of activity-induced Arc turnover. Our findings indicate that NO promotes Arc degradation through S-Nitrosylation at Cys96 and Cys159. Mutation of these residues attenuated NO-dependent Arc turnover, prolonged Arc abundance, and enhanced AMPA receptor internalization. Thus, nNOS-derived NO may function as a temporal regulator that fine-tunes the duration of Arc-dependent synaptic signaling and helps maintain AMPA receptor homeostasis.

Introduction

Long-term memory formation requires that transient patterns of neuronal activity be converted into lasting structural and functional changes at synapses, a process supported by activity-dependent gene expression¹⁻⁴. Among the immediate-early genes, activity-regulated cytoskeleton-associated protein (Arc) plays a central role in synaptic plasticity and memory^{5,6}. Its mRNA and protein levels are regulated at multiple stages from transcription and local translation to trafficking and protein turnover⁶⁻⁹. Unlike encoding a transcription factor, Arc functions as a synaptic effector that acted directly at synapse coupling neuronal activity to local changes^{6,10}.

A defining feature of Arc is its transient expression profile^{6,8,11}. Following neuronal activation, Arc protein is rapidly induced but subsequently declines over the ensuing hours¹². This temporal regulation is critical because Arc promotes AMPA-type glutamate receptor (AMPA) endocytosis through interactions with the endocytic machinery¹³⁻¹⁵ and mediates homeostatic synaptic scaling¹⁵⁻¹⁷ and metabotropic glutamate receptor-dependent long-term depression¹⁸⁻²⁰. Consequently, prolonged Arc persistence extends AMPAR internalization beyond the appropriate time window and alter receptor homeostasis^{21,22}; impaired Arc turnover can lead to its pathological accumulation, which has been in learning and cognitive deficits associated with autism and intellectual disability^{21,23}. Mechanisms controlling Arc transcription and translation have been extensively studied^{18,24}, the activity-dependent signals that limit Arc protein persistence after induction remain incompletely understood.

Arc turnover is regulated by several pathways, including ubiquitin-dependent proteasomal degradation by UBE3A^{21,25}, Triad3A²⁶, and CHIP²⁷, as well as autophagy²⁸, and GSK3-dependent phosphorylation²⁹. Although these pathways define the effectors of Arc removal, the activity-dependent signals that drive their timely engagement remains unidentified.

Synaptic activation also triggers calcium influx through NMDA receptors (NMDARs), which activate neuronal nitric oxide synthase (nNOS) through its association with the postsynaptic scaffolding protein PSD-95^{30,31}. Activated nNOS generates nitric oxide (NO), a diffusible signaling molecule that regulates neuronal excitability, synaptic transmission, and plasticity³²⁻³⁵. In addition to signaling through soluble guanylyl cyclase, NO modifies reactive cysteine residues through S-Nitrosylation, a reversible post-translational modification³⁶ that alters protein conformation, protein interactions, localization, and stability³⁷, regulate the ubiquitination and proteasomal processing of many neuronal proteins^{38,39}, suggesting that it may provide a route through which activity-dependent NO signaling influences protein turnover.

Here, we investigated whether nNOS-derived NO contributes to the temporal regulation of Arc protein abundance. Using neuronal stimulation paradigms, quantitative proteomics, pharmacological manipulation, nNOS-deficient mice, and cysteine-mutant Arc constructs, we showed that nNOS/NO signaling promotes the late decline in Arc protein. Cys96 and Cys159 are the major S-Nitrosylation sites of Arc, and that these residues are required for GSNO-induced Arc protein turnover. Furthermore, we found that S-Nitrosylation of Arc at Cys96 and Cys159 regulates surface trafficking of AMPA receptors. Together, these results implicate NO-dependent S-Nitrosylation in driving the timely turnover of Arc following neuronal activation.

Results

Neuronal activity induces dynamic changes of Arc protein level

To characterize stimulus-dependent changes in Arc protein level, we employed several established neuronal activation paradigms, including tetrodotoxin washout (TTX washout)^{8,12,40}, glutamate application, and NMDA treatment (Fig. S1 and Fig. 1A, B). Across these paradigms, neuronal stimulation induced a rapid increase in Arc protein levels. Arc abundance peaked approximately 2 h after stimulation and subsequently declined at later time points, including 6 and 24 h (Fig. 1A, B). Similar temporal dynamics were observed following different stimulation protocols (Fig. S1A-E), indicating that the dynamic changes of Arc protein level are a shared response to multiple forms of neuronal activation. We next examined whether a similar temporal pattern occurs *in vivo* following an aversive stimulus. Wild-type (WT) mice were subjected to acute foot shock (Fig. 1C), which evokes neuronal activation in fear-conditioning-related brain regions^{41,42}. Consistent with the *in vitro* findings, Arc protein levels increased after foot shock, reached a maximum at approximately 2 h, and declined by 24 h (Fig. 1D, E). Immunofluorescence analysis further revealed corresponding changes in Arc signal in the CA1 region of the hippocampus (Fig. 1F, G). Together, these findings support the well-established activity-dependent induction of Arc^{8,12,41,43} and demonstrate that Arc protein abundance declines after reaching its post-stimulation peak under both *in vitro* and *in vivo* conditions. However, the molecular mechanisms that underlie this late-phase reduction in Arc protein levels remain incompletely understood.

Proteomic analyses identify nNOS as a candidate regulator of the late decline in Arc abundance

To identify candidate pathways associated with activity-dependent dynamic changes in Arc abundance, we performed time-course proteomic profiling of cultured neurons following NMDA stimulation (Fig. 2A). Differentially expressed proteins across the time course were used to construct a protein–protein interaction (PPI) network (Fig. 2B, C). Using a restart-based random-walk analysis with NMDA receptor subunits GluN1, GluN2A, and GluN2B as seed nodes to prioritize proteins located in close network proximity to NMDAR signaling components, the top-ranked proteins included

postsynaptic scaffolding and signaling proteins (PSD95, Shank3, SAPAP1, SynGAP)^{44,45}, phosphorylation-dependent signaling proteins (PKA-C α , PP1 γ , p59-Fyn)^{46,47}, calmodulin-like protein 4 (CALML4), AMPA receptor subunit GluA1 and neuronal nitric oxide synthase (nNOS) (Fig. 2D and Fig. S2). nNOS emerged as particularly significant because its coupling to NMDAR-associated signaling provides a potential link between activity-dependent calcium influx and nitric oxide (NO) production^{31,48}. Moreover, previous work in our lab showed that NO promotes p35 and p39 protein degradation under physiological and pathological conditions^{38,39}. We therefore examined whether nNOS/NO signaling contribute to the activity-dependent regulation of Arc protein stability. Under the same NMDA-stimulation conditions, Arc abundance declined during the later phase of the response, whereas nNOS abundance increased or recovered over this interval (Fig. 2E, F). This temporal association, combined with the network analysis, nominated nNOS as a candidate regulator of Arc protein abundance.

We next examined whether nNOS influences the temporal dynamic profile of Arc *in vivo*. Wild-type (WT) and nNOS-knockout (nNOS-KO) mice were subjected to acute foot shock, followed by time-course quantitative proteomic analysis of hippocampal tissue (Fig. 3A). Hierarchical clustering identified a distinct group of rapid-response synaptic proteins (Cluster 1) that underwent swift decay in WT mice (Fig. S3A-C). As predicted, the rapid decline of Arc within this dynamic cluster was blocked in nNOS-KO mice (Fig. 3B-D). Collectively, these findings point to nNOS as a regulator of the late reduction in Arc protein abundance following neuronal activation.

NO signaling promotes Arc degradation in a proteasome-dependent manner

Next, to examine whether NO signaling directly regulates Arc protein abundance, we treated cortical neurons and hippocampal slices with the NO donor S-Nitrosoglutathione (GSNO). GSNO treatment resulted in a time-dependent reduction in basal Arc protein levels (Fig. 4A, B and Fig. S4A, B). To distinguish whether reduced protein abundance resulted from either diminished protein synthesis or enhanced

protein degradation, we compared Arc turnover in cultured cortical neurons treated with GSNO or the protein synthesis inhibitor cycloheximide (CHX). Arc protein levels declined substantially more rapidly following GSNO treatment than following CHX treatment (Fig. 4C, D), suggesting that NO signaling promotes Arc degradation. Consistent with this interpretation, the proteasome inhibitor MG132 blocked the GSNO-induced reduction of Arc levels (Fig. S5A, B) and GSNO treatment increased the ubiquitination of endogenous Arc in cultured neurons (Fig. S5C). These findings indicate that NO-induced Arc degradation depends on proteasome activity. Conversely, application of the NOS inhibitor L-NAME caused a significant accumulation of Arc protein within 1 to 4 h (Fig. 4E, F and Fig. S4C, D). To further validate whether endogenous NO signaling regulates Arc stability *in vivo*, we compared Arc protein levels in the hippocampus of nNOS-KO mice. Consistent with the pharmacological data, Arc protein levels were significantly elevated in the hippocampus of nNOS-KO mice relative to WT controls (Fig. S4E, F).

Synaptic plasticity relies on a transient burst of Arc expression^{6,8}, and its subsequent rapid degradation is essential to maintain this narrow temporal window. To assess whether NO signaling mediates Arc degradation during neuronal activity, we applied acute KCl depolarization to cultured neurons from WT and nNOS-KO mice. Upon KCl-induced depolarization, Arc protein levels peaked at 2 h and then declined sharply at 6 h in the control group, whereas this late-phase decline was markedly impaired in nNOS-KO neurons, resulting in sustained Arc accumulation (Fig. S4G, H). In line with these findings, the inhibition of NOS with L-NAME also blocked the late-phase degradation of Arc induced by either glutamate (Fig. S4I, J) or NMDA (Fig. 4 G, H) stimulation in WT neurons. Taken together, these findings demonstrate that NO signaling negatively regulates Arc protein stability by promoting its proteasome-dependent degradation during the late-phase of synaptic activation.

NO S-Nitrosylates Arc at Cys96 and Cys159

Next, we detected the underlying mechanism by which NO signaling promotes Arc degradation. Previous work in our lab showed that NO can regulate protein stability via S-Nitrosylation^{38,39}, a covalent modification of cysteine thiols^{37,49}. Thus, we firstly investigated whether Arc could undergo S-Nitrosylation by HEK293T cells expressing wild-type Arc (Arc-WT) treated with the NO donor GSNO and subjecting them to biotin switch assay (BSA). Markedly increased Arc S-Nitrosylation level following GSNO treatment (Fig. 5A, B). To determine whether this modification occurs under physiological conditions, we examined endogenous S-Nitrosylated Arc (SNO-Arc) in mouse brain tissue. Importantly, we detected robust SNO-Arc in WT brain tissue, whereas SNO-Arc was significantly reduced in nNOS-KO mice, suggesting that endogenous nNOS activity is a major contributor to Arc S-Nitrosylation in the brain (Fig. 5C).

To identify potential S-Nitrosylation sites, we analyzed the distribution of cysteine residues within the Arc protein. Sequence alignment of Arc across multiple species (mouse, rat, and human) revealed five highly conserved cysteine residues (Fig. S6 and Fig. 5D). We then generated individual cysteine-to-alanine mutants for each site (Arc-CxxxA mutants). HEK293T cells expressing Arc-WT, individual cysteine mutants, or combined mutants were treated with GSNO and analyzed by BSA. Whereas Arc-WT was markedly S-Nitrosylated upon GSNO treatment, mutation of Cys96 or Cys159 substantially reduced the S-Nitrosylation signal, with combined mutation causing further reduction Arc S-Nitrosylation (Fig. 5E, F). These findings identify these two cysteines as major S-Nitrosylation sites on Arc.

S-Nitrosylation of Arc at Cys96 and Cys159 contributes to NO-dependent Arc degradation and regulates surface GluA1 trafficking

To determine whether NO-dependent Arc degradation is mediated by S-Nitrosylation at Cys96 and Cys159, we employed a Tet-Off system to control the expression of Arc-WT and Arc-C96A&C159A double mutant in HEK293T cells. GSNO treatment significantly accelerated Arc-WT degradation, whereas Arc-C96A&C159A

degradation remained largely unaffected (Fig. 6A, B). These results indicated that S-Nitrosylation at Cys96 and Cys159 promotes NO-dependent Arc degradation.

Arc is known to promote the internalization of AMPA-type glutamate receptor (AMPA)^{6,13}. We therefore hypothesized that sustained Arc accumulation would suppress surface AMPAR levels^{21,22}. Consistent with this hypothesis, L-NAME treatment increased Arc abundance and reduced surface GluA1 levels in cultured neurons (Fig. S7A, B). To determine whether the surface GluA1 suppression is mediated by Arc S-Nitrosylation and subsequent degradation, we co-transfected HEK293T cells with a Super Ecliptic pHluorin-tagged GluA1 subunit (SEP-GluA1)^{50,51} and either empty vector, Arc-WT, or Arc-C96A&C159A. Arc-WT expression reduced surface SEP-GluA1 fluorescence relative to empty vector controls (Fig. 6C-E). GSNO treatment increased surface SEP-GluA1 fluorescence in cells expressing Arc-WT, consistent with reduced Arc abundance. In contrast, GSNO did not restore surface SEP-GluA1 fluorescence in cells expressing Arc-C96A&C159A (Fig. 6C-E). Our results support a model in which Cys96 and Cys159 contribute to NO-dependent Arc degradation, thereby regulating the AMPAR surface trafficking.

Discussion

In this study, we identify nNOS-derived nitric oxide (NO) as a contributor to the late decline in Arc protein abundance following neuronal activation. Across multiple *in vitro* stimulation paradigms and acute foot-shock experiments in WT and nNOS-KO mice, genetic or pharmacological disruption of nNOS/NO signaling attenuated the late reduction in Arc protein levels. In addition, mutational analyses implicated Cys96 and Cys159 as Arc S-Nitrosylation sites and mediated NO-induced protein degradation. Moreover, Arc S-Nitrosylation at Cys96&159 regulated GluA1 trafficking. Together, these findings support a model in which NO signaling participates in the fine temporal regulation of Arc after neuronal activation.

Although the transcriptional regulation of immediate-early genes (IEGs) has been extensively studied^{40,52,53}, less is known about how their protein products are cleared

after completing their transient functions. A recent study identified the midnolin–proteasome pathway as a mechanism underlying the rapid turnover of nuclear IEG transcription factors, including c-Fos and EGR1⁵⁴. However, whole-brain mapping has revealed substantial differences in the spatiotemporal expression patterns of Arc and c-Fos⁴², despite their frequent use as IEG markers. Moreover, Arc functions primarily at synapses^{5,9}, whereas c-Fos acts as a nuclear transcription factor^{52,55}. Their distinct subcellular contexts suggest that the turnover of Arc and nuclear IEGs may be governed by different regulatory mechanisms. Our findings identify nNOS/NO signaling and Arc S-Nitrosylation as one potential mechanism linking neuronal activity to the lifetime of a synaptic IEG effector protein.

Neuronal plasticity depends not only on the magnitude of activity-induced protein expression but also on the duration for which these proteins remain functionally available^{22,56,57}. This temporal regulation is shaped by molecular processes operating across multiple timescale, from rapid post-translational modifications to dynamic changes in protein synthesis and degradation, which together reshape the local proteome over minutes to hours⁵⁸. Arc provides a representative example of this temporal regulation: its activity-induced expression is followed by relatively rapid protein turnover, thereby fine-tuning the duration of its functional availability. Our findings suggest that S-Nitrosylation provides a mechanism for regulating Arc protein stability. NO is known to regulate neuronal plasticity primarily through the sGC–cGMP–PKG signaling cascade and protein S-Nitrosylation⁵⁹. Whereas NO–cGMP signaling has been implicated in the activity-dependent transcriptional induction of IEGs, including Arc induction⁶⁰. Our results suggest that nNOS-derived NO may also influence the later phase of the Arc response by promoting a reduction in Arc abundance. Thus, NO signaling may contribute to the temporal profile of Arc through distinct downstream mechanisms acting at different stages after neuronal activation.

Our mutagenesis experiments identified Cys96 and Cys159 as key S-Nitrosylation sites contributing to NO-induced Arc degradation. Notably, Cys159 has recently been implicated in redox-dependent Arc turnover: disulfide crosslinking involving Cys34 and Cys159 promotes Arc oligomerization and subsequent degradation through an

Hsp70–CHIP-dependent mechanism⁶¹. Although disulfide formation and S-Nitrosylation are distinct modifications, their convergence at Cys159 suggests that this residue may serve as a redox-sensitive regulatory site. S-Nitrosylation can alter protein conformation, folding, protein–protein interactions, and subcellular localization^{37,62}, modification of Cys96 and Cys159 by NO may influence the conformational state of Arc and thereby increase its susceptibility to recognition by degradation machinery. Direct site-resolved mass spectrometry, together with structural and interaction studies, will be important for establishing whether these residues are endogenously S-Nitrosylated and how their modification affects Arc recognition by degradation pathways.

Previous studies have established that Arc clearance relies on ubiquitin-dependent^{21,25-27} and ubiquitin-independent⁶³ proteasomal pathways. In addition, post-translational modifications such as phosphorylation can regulate Arc turnover, with GSK3-dependent phosphorylation promoting subsequent degradation²⁹. Notably, S-Nitrosylation has also been shown to modulate the ubiquitin–proteasome system, with increasing evidence revealing that this redox modification can influence protein ubiquitination and proteasomal turnover in a context-dependent manner⁶⁴. In line with this framework, we found that GSNO treatment markedly increased Arc ubiquitination, providing further support for the involvement of the ubiquitin–proteasome system in NO-dependent regulation of Arc. These findings raise the possibility that S-Nitrosylation facilitates the recognition or processing of Arc by the ubiquitin–proteasome system. Identifying the E3 ubiquitin ligases and associated quality-control factors that recognize this redox-regulated state will be an important next step in defining how activity-dependent signaling regulates the lifetime of Arc.

We found that GSNO failed to increase surface GluA1 levels in cells expressing the S-Nitrosylation-deficient Arc mutant, which suggests that NO-dependent Arc regulation is functionally linked to the recovery of surface GluA1. S-Nitrosylation-mediated Arc clearance may therefore constrain the duration of Arc-dependent AMPA receptor internalization, whereas impaired NO signaling could prolong Arc persistence and perturb synaptic receptor homeostasis. Dysregulated Arc expression and turnover have

299 been associated with synaptic dysfunction across several neurological disorders^{21,65,66},
300 while dysregulated NO signaling and aberrant protein S-Nitrosylation are also
301 implicated in neurodegenerative disease^{37,67,68}. Whether disruption of the NO–Arc
302 pathway contributes to pathological alterations in synaptic receptor homeostasis
303 remains to be determined.

304 Together, our findings identify S-Nitrosylation as a key mechanism contributing to Arc
305 degradation following its initial activity-dependent induction. By promoting ubiquitin-
306 dependent degradation of Arc, NO signaling constrains the duration of Arc activity and
307 thereby limits the persistence of Arc-dependent AMPA receptor trafficking. These
308 findings suggest that the regulation of Arc depends not only on its activity-dependent
309 induction but also on the timely clearance. More broadly, our study suggests that redox-
310 dependent post-translational regulation may provide a molecular mechanism for
311 terminating transient synaptic programs and maintaining the appropriate duration of
312 activity-induced plasticity.

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Materials and methods

Animals. Age-matched (8–12 weeks) male and female nNOS-knockout (nNOS-KO) mice and their littermate wild-type (WT) controls, derived from heterozygous breeding pairs, were utilized in this study. The nNOS-KO mice were a generous gift from Professor Dongya Zhu (Nanjing Medical University). All mice were housed in a temperature-controlled room with a 12/12 h light/dark cycle, with humidity controlled as 55%, provided with food and water ad libitum. All animal-related procedures, including surgery and care, adhered to approved protocols by the Animal Care and Use Committees at Shanghai Jiao Tong University School of Medicine (SYXK(Hu)2018-0027).

Reagents and plasmids. All chemicals were purchased from Sigma unless stated otherwise. Antibodies anti-Arc for Western blotting (1:1,000, Abcam, ab183183) and anti-Arc for immunofluorescence staining (1:1,000, Synaptic Systems, 156003), anti-nNOS (1:1,000, CST, 4231), anti-GluR1-N terminus, clone RH95 (1:1,000, MAB2263), anti-c-Myc (1:1,000, Santa Cruz, sc-40), anti-GAPDH (1:5,000, Proteintech, 60004-1-Ig), anti-Ubiquitin (1:1,000, Huabio, ET1609-21) and anti- β -actin (1:5,000, A5441) as well as horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were from Beyotime. The mouse Arc plasmid was a generous gift from Dr. Jacque Ip at The Chinese University of Hong Kong, the sep-GluA1 plasmid was a generous gift from Professor Wei-Qun Fang at Shanghai Jiao Tong University School of Medicine and the Tet-off plasmid was purchased from Miaoling Biology.

Fear conditioning (Foot shock). Animals were trained in standard conditioning chambers with stainless-steel grid floors for foot shock administration. Following a 180 s initial habituation period in the chamber, animals were presented with three tone-shock pairings. Each pairing consisted of an auditory conditioned stimulus (30 s, 5 kHz, 85 dB) that co-terminated with a mild foot shock (0.75 mA, 2s)^{41,69}. The inter-trial interval between tone-shock presentations was 210 s. The conditioning chamber was thoroughly cleaned with 70% ethanol between animals. 0 h controls received no exposure to the conditioning chamber and remained in standard housing conditions until they were sacrificed.

LC-MS/MS analysis and protein identification. Each sample was analyzed using a nano-UPLC (Vanquish Neo) coupled to an Astral Zoom instrument (Thermo Scientific) with a nano-electrospray ion source. Separation was performed using a reversed phase column (EASY-Spray™ HPLC (150 μ m $\times$ 15 cm), Thermo Scientific, USA). Mobile phases were H₂O with 0.1% FA (phase A) and 80% ACN with 0.1% FA (phase B).

Data-Independent Acquisition (DIA) was performed with positive ion detection. The parameters were set as follows: electrospray voltage at 1,800 V, temperature at 280 °C; MS1 scan range of 380–980 m/z with a resolution of 240k, AGC target of 500%, and maximum injection time of 3 ms; MS2 scan range of 150–2,000 m/z with a maximum injection time of 3 ms, isolation window of 2 Th, RF-lens at 40%, MS2 activation type of HCD, normalized collision energy of 25%, and a cycle time of 0.6 s.

The raw files were processed using DIA-NN software (v2.2.0) and the built-in Pulsar search engine. MS spectra lists were searched against their species-level UniProt FASTA databases (10090_all.fasta). The parameters for analyses were set as follows: trypsin enzyme; up to 2 missed cleavages; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine and acetylation of protein N-terminus as variable modifications. Peptide identification was performed with an initial precursor mass deviation of up to 20 ppm and a fragment mass deviation of 20 ppm. The false discovery rate (FDR) was set to 0.01 for both PSM and peptide levels. At least 2 unique peptides (peptides that only match 1 protein sequence) or razor peptides (peptides that might match to more than 1 protein sequences) per protein group was required for protein identification. All other parameters were reserved as default.

Site-directed mutagenesis. Site-directed mutagenesis was performed with oligonucleotide primers designed to substitute the corresponding cysteine residue(s) with alanine residue(s) using overlap PCR. All mutations were verified by plasmid DNA sequencing.

Cell culture and plasmid transfection. HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Royacel) plus antibiotics. Cells

were transfected with expression constructs using Lipo8000™ reagent (Beyotime). Primary cortical neurons were prepared from embryonic day (E) 17 mouse embryos, seeded on cultured plates coated with poly-L-lysine (Sangon Biotech) and maintained in Neurobasal medium supplemented with 2% B27 (Thermo Scientific) and 2 mM Glutamax (Thermo Scientific). For drug treatment, 100 μM GSH/GSNO, 600 μM L-NAME, 100 μM glutamate, 50 μM NMDA, 0.5 μM TTX (Tauto) and 50 mM KCl, was added to the cultured cells unless stated otherwise.

Biotin switch assay. The biotin switch assay was performed to detect protein S-Nitrosylation^{38,39,70}. Briefly, HEK293T cells overexpressing Arc or its cysteine mutants were lysed in HENT buffer (250 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine, and 1% Triton X-100, pH = 7.7). The cell lysates were then incubated with 10 mM S-methyl methanethiosulfonate (MMTS) for 20 min at 50 °C to block free cysteine, and excess MMTS was removed by three passes through acetone precipitation. The samples were then incubated with 5 mM ascorbate to specifically reduce S-Nitrosylated cysteine to free cysteine and 0.5 mM N-[6-(biotinamido)hexyl]-30-(20-pyridyldithio) propionamide (biotin-HPDP) at room temperature with rotation overnight to label the reduced cysteines. Unreacted biotin-HPDP was removed by acetone precipitation, and the biotinylated samples were then resuspended and incubated with 40 μl NeutrAvidin agarose for 1 h. The pellets were washed five times with neutralization buffer with 0.6 M NaCl, eluted by 2 × SDS sample buffer and subjected to Western blot analysis.

Surface biotinylation assay. Primary cortical neurons were treated with L-NAME for different time was washed in ice-cold Dulbecco's phosphatebuffer (DPBS) and subsequently incubated with 1 mg/ml EZ-link-sulfo-NHS-Biotin (Pierce) on ice for 0.5 h. According to previously described protocols³⁸, the biotin solution was removed, and the reaction was quenched by the 50 mM Tris-HCl (pH 7.4). Neurons were then lysed with radio immunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors. Non-solubilized material was removed by centrifugation at 13,200 rpm for 10 min. Biotinylated proteins were separated from non-biotinylated proteins by incubation with streptavidin agarose

(Pierce) overnight at 4 °C. Beads were washed with the lysis buffer, and the adsorbed proteins were eluted with 2 × SDS sample buffer.

Immunohistochemistry. Animals were perfused with 4% paraformaldehyde (Servicebio). Brains were dissected and post-fixed in 4% paraformaldehyde for 24 h at 4 °C and then transferred to DPBS stored at 4 °C. Embedding was performed in 3% agarose at 4 °C until slicing. Forty-micrometer coronal sections were collected serially using a VT1200 Vibratome (Leica, Germany). Pre-incubation with blocking buffer containing 0.1% Triton X-100 (Sangon Biotech) and 3% BSA (Beyotime). Sections were then incubated in anti-Arc primary antibodies for 24 h at 4 °C followed by incubation with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:500, Invitrogen, A-11008) for 2 h at room temperature. Both primary and secondary antibodies were diluted in blocking buffer. Nuclear staining was performed using DAPI (1:1,000, D9542) and sections were mounted on slides and coverslip. Images were taken using Nikon AX laser scanning microscopes.

Flow cytometry analysis. To evaluate SEP-GluA1 expression, HEK293T cells co-transfected with SEP-GluA1 and either an empty vector or Arc (WT or mutants) were treated with GSH and GSNO, and subsequently analyzed on an Attune NxT Flow Cytometer (Thermo Scientific, USA). SEP fluorescence was excited using a 488 nm laser and captured with a 530/30 nm filter. Intact cells were gated using forward (FSC) and side scatter (SSC), followed by doublet exclusion. The SEP gate was calibrated based on cells treated with lipofectamine only, which are expected to be SEP-negative, ensuring accurate distinction between positive and negative SEP signals. FlowJo software (v10.8.1) was used to determine the median fluorescence intensity (MFI) of SEP-positive cells.

Protein-protein interaction (PPI) network construction and random walk with restart (RWR) analysis. Using the STRING database (<https://string-db.org/>), DEGs was performed to select interaction relationships with confidence scores greater than or equal to 0.9 to construct PPI networks. The NMDA receptor subunits (GluN1, GluN2A,

and GluN2B) in the PPI network were defined as seed genes, and Random Walk with Restart (RWR) analysis of the PPI network was performed using the R packages with a set restart probability of 0.7. Column-normalization was used to normalize the adjacency matrix of the network graph, and the topological score (affinity coefficient) between each gene and the seeds were obtained after RWR analysis.

Statistical analysis. Data are presented as mean $\pm$ standard error of the mean (s.e.m.) from at least three independent experiments. Prior to parametric testing, data from all groups were evaluated to confirm normal distribution and equal variance. For comparisons between two groups, statistical significance was determined using the two-tailed Student's *t*-test. For comparisons involving three or more groups with a single independent variable, one-way ANOVA with Dunnett's or Tukey's post hoc test was performed. For data that failed to meet the assumption of normality, the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. For experiments assessing the effects of two independent variables, two-way ANOVA with Dunnett's post hoc test was performed. For comparing two groups at corresponding time points, unpaired Welch's *t*-test was applied to account for unequal variances; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and n.s., not significant (*p* $\geq$ 0.05).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PZ, AC and YY conceived and supervised the project. PZ, JW, LW, JW, YL, JL, XG, YL and KZ performed the experiments. PZ, AC, YY, YH and JW wrote the manuscript with input and help from other members from PZ lab. All authors contributed to data interpretation and data analysis.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure Legend

Fig.1 | Neuronal activity induces dynamic changes of Arc protein level *in vitro* and

***in vivo*.** (A, B) Acute NMDA exposure rapidly altered Arc protein levels *in vitro*. Cultured cortical neurons were treated with NMDA for 5 min, returned to conditioned culture medium, and collected at the indicated time points (0, 0.25, 0.5, 2, 6, and 24 h) for Western blot analysis. Values were normalized to the 0 h group and shown as mean $\pm$ s.e.m. from $n = 3$ independent experiments. *** $p = 0.0002$ (0 h), *** $p = 0.0002$ (0.25 h), *** $p = 0.0003$ (0.5 h), *** $p = 0.0004$ (6 h), *** $p < 0.001$ (24 h) versus 2 h group; one-way ANOVA with Dunnett's post hoc test. (C) Schematic of fear conditioning (FC) assays. This panel was generated using BioRender.com. (D–G) FC drives rapid temporal dynamics of Arc protein in the hippocampus *in vivo*. Wild-type mice were subjected to FC and sacrificed at 2 h and 24 h after conditioning; hippocampal tissues were then harvested for Western blot and immunofluorescence analyses. (D) Representative Western blots. (E) Values are normalized to the control (0 h) group and represent mean $\pm$ s.e.m. from $n = 3$ independent experiments. * $p = 0.0171$, ** $p = 0.0032$ versus 2 h group; one-way ANOVA with Dunnett's post hoc test. (F) Representative immunofluorescence images of the hippocampal CA1 region, Scale bar, 100 μ m. (G) Quantitative analysis of Arc mean fluorescence intensity (MFI). Values are represented mean $\pm$ s.e.m. $n = 4$ independent experiments. *** $p < 0.001$ versus 2 h group; one-way ANOVA with Dunnett's post hoc test.

Fig.2 | Dynamic proteome network prioritizes nNOS as a candidate regulator of

Arc protein changes. (A) Schematic overview of the experimental data processing workflow. This panel was generated using BioRender.com. (B) Volcano plot of the identified differentially expressed proteins (DEPs) at each time point following NMDA stimulation. For each protein, the log fold change relative to the 0 h group is plotted against the adjusted p value. Different colors indicate density of data points. (C) Venn diagram showing the number of identified DEPs at 2 h, 6 h and 24 h for the three biological replicates per time point. (D) The core signalosome sub-network comprising the top 10 ranked proteins. A global protein-protein interaction (PPI) network was

constructed from the union of all DEPs, followed by Random Walk with Restart (RWR) analysis to identify key targets. Red nodes in the inner circle represent the fixed seed nodes (GluN1, GluN2A, GluN2B). In the outer circle, the gold node highlights the highly ranked target nNOS, and light blue nodes indicate other top functional interactors. Node size is directly proportional to the RWR score, with larger nodes indicating a stronger functional association with the seed nodes. (E, F) Inverse temporal dynamics of Arc and nNOS protein levels following NMDA stimulation. Representative Western blot images (E) and quantitative time-course analysis (F) of Arc (red line, left y-axis) and nNOS (yellow line, right y-axis) protein levels. All values are normalized to the respective 0 h group and shown as mean $\pm$ s.e.m. from $n = 3$ independent experiments.

Fig.3 | nNOS deficiency abolishes the activity-induced temporal response of Arc *in vivo*. (A) Schematic overview of the experimental data processing workflow. WT and nNOS-KO mice were subjected to fear conditioning, followed by hippocampal tissue collection and proteomic analysis. This panel was generated using BioRender.com. (B) Impaired temporal dynamics of activity-induced proteins in nNOS-KO mice. The heatmap shows cluster1 of representative synapse- and neuron-related proteins (Arc was highlighted) that are dynamically regulated in WT at 0, 2, and 24 h after fear conditioning. In nNOS-KO mice, this dynamic response is blunted. (C) Representative Western blot images of Arc across time points in WT and nNOS-KO mice. Arc displays temporal dynamics expression in WT but the response is lost in nNOS-KO. (D) Quantification of Arc protein levels. Values are normalized to the 0 h group and shown as mean $\pm$ s.e.m. from $n = 3$ independent experiments. $*p = 0.0185$, $***p = 0.0009$ versus the 2 h in the WT group, no significant temporal alterations were observed in the nNOS-KO group; two-way ANOVA (Genotype $\times$ Time) with Dunnett's post hoc test.

Fig.4 | NO promotes the degradation of Arc protein. (A, B) GSNO-derived NO downregulates Arc protein expression. Cultured cortical neurons were treated with the NO donor GSNO for the indicated time points, and then subjected to Western blot

analysis. (B) Quantitative analysis of Arc protein expression. All values are normalized

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Fig.5 | NO S-Nitrosylates Arc at Cys96&159. (A, B) Arc is S-Nitrosylated *in vitro*.

(A) HEK293T cells expressing wild-type Arc (Arc-WT) was exposed to GSNO for 30

min, lysed and subjected to the biotin switch assay. (B) Quantitative analysis of Arc S-

Nitrosylation levels. Values are normalized to the “GSNO” group and shown as mean

± s.e.m. from n = 4 independent experiments. *** $p < 0.001$; unpaired two-tailed

Student’s *t*-test. (C) Arc is S-Nitrosylated *in vivo*. Brain lysates prepared from adult WT

and nNOS-KO mice were subjected to the biotin-switch assay. (D-F) Cys96 and Cys159

are the predominant S-Nitrosylation sites on Arc. (D) Predicted structural model of the

Arc protein. (E) HEK293T cells expressing Arc-WT or its cysteine mutants were

exposed to GSNO for 30 min, lysed and subjected to the biotin switch assay. (F)

Quantitative analysis of Arc S-Nitrosylation levels. Values are normalized to the “Arc-

WT + GSNO” group and shown as mean ± s.e.m. from n ≥ 3 independent experiments.

*** $p < 0.001$ (WT + GSH), *** $p = 0.0008$ (C96A + GSNO), *** $p < 0.001$ (C159A +

GSNO).

GSNO), *** $p = 0.0001$ (C96A& C159A + GSNO); one-way ANOVA with Dunnett's post hoc test.

Fig.6 | Arc S-Nitrosylation at Cys96&159 mediates NO-dependent regulation of surface AMPA receptor availability. (A, B) Cys96 and Cys159 mediate NO-dependent Arc degradation. (A) Representative Western blots showing the degradation of Arc-WT and the C96A&C159A mutant. At 24 h post-transfection with Arc-WT or its C96A&C159A mutant, cells were exposed to the NO donor GSNO or GSH in the presence of Dox. (B) Quantitative analysis of Arc protein levels. Values are normalized to the WT (GSH treatment) group and shown as mean $\pm$ s.e.m. from $n = 5$ independent experiments. ** $p = 0.0020$, *** $p = 0.0002$; one-way ANOVA with Tukey's post hoc test. (C-E) Arc S-Nitrosylation at C96 and C159 mediates NO-regulated surface GluA1 expression. (C) Representative immunofluorescence images of SEP-GluA1 fluorescence intensity in HEK293T cells co-transfected with SEP-GluA1 and a Tet-Off inducible Arc-WT or Arc-C96A&C159A construct, followed by treatment with GSH or GSNO in the presence of Dox. Scale bar, 50 μ m. (D) Representative flow cytometry profiles showing SEP-GluA1 fluorescence. (E) Quantitative analysis of SEP-GluA1 median fluorescence intensity (MFI). Values are represented mean $\pm$ s.e.m. $n = 3$ independent experiments. ** $p = 0.0096$ (GSH-WT vs GSNO-WT), *** $p < 0.001$ (GSH-vector vs GSH-WT), *** $p < 0.001$ (GSH-vector vs GSH-C96A&C159A), *** $p < 0.001$ (GSNO-vector vs GSNO-WT), *** $p < 0.001$ (GSNO-vector vs GSNO-C96A&C159A), *** $p = 0.0003$ (GSNO-WT vs GSNO-C96A&C159A); one-way ANOVA with Tukey's post hoc test.

Fig.1 Neuronal activity induces dynamic changes of Arc protein level *in vitro* and *in vivo*

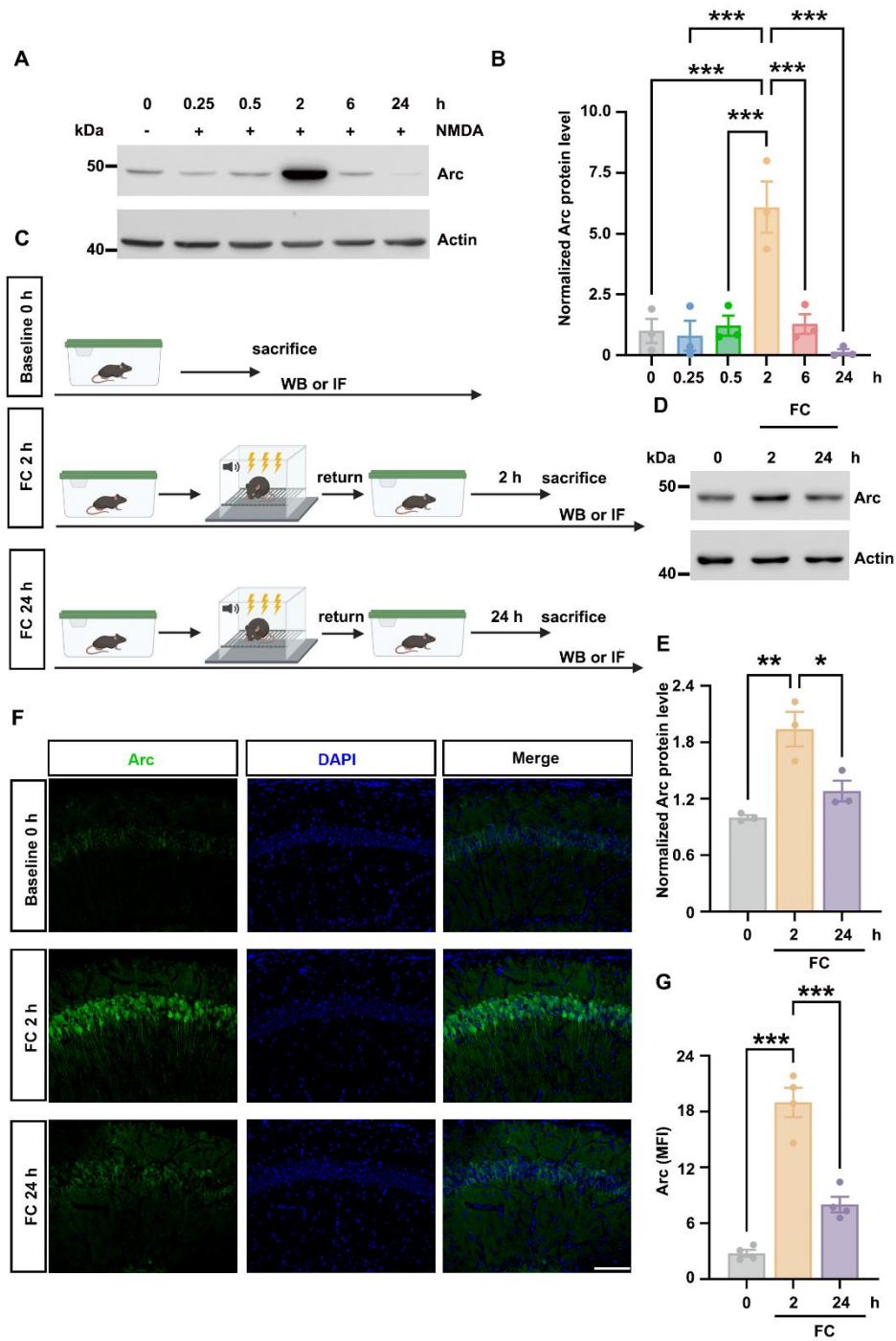


Fig.2 Dynamic proteome network prioritizes nNOS as a candidate regulator of Arc protein changes

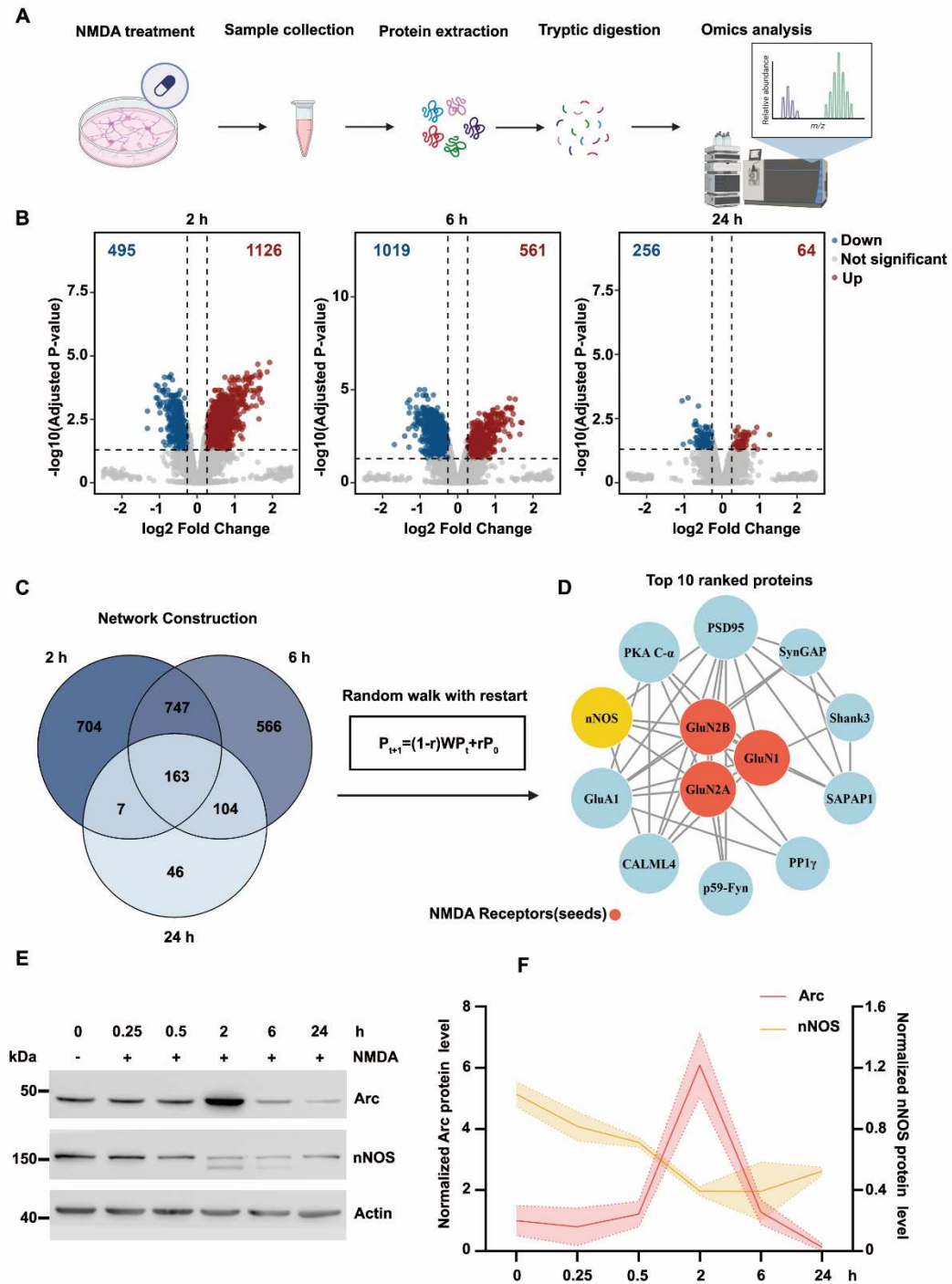


Fig.3 nNOS deficiency abolishes the activity-induced temporal response of Arc *in vivo*

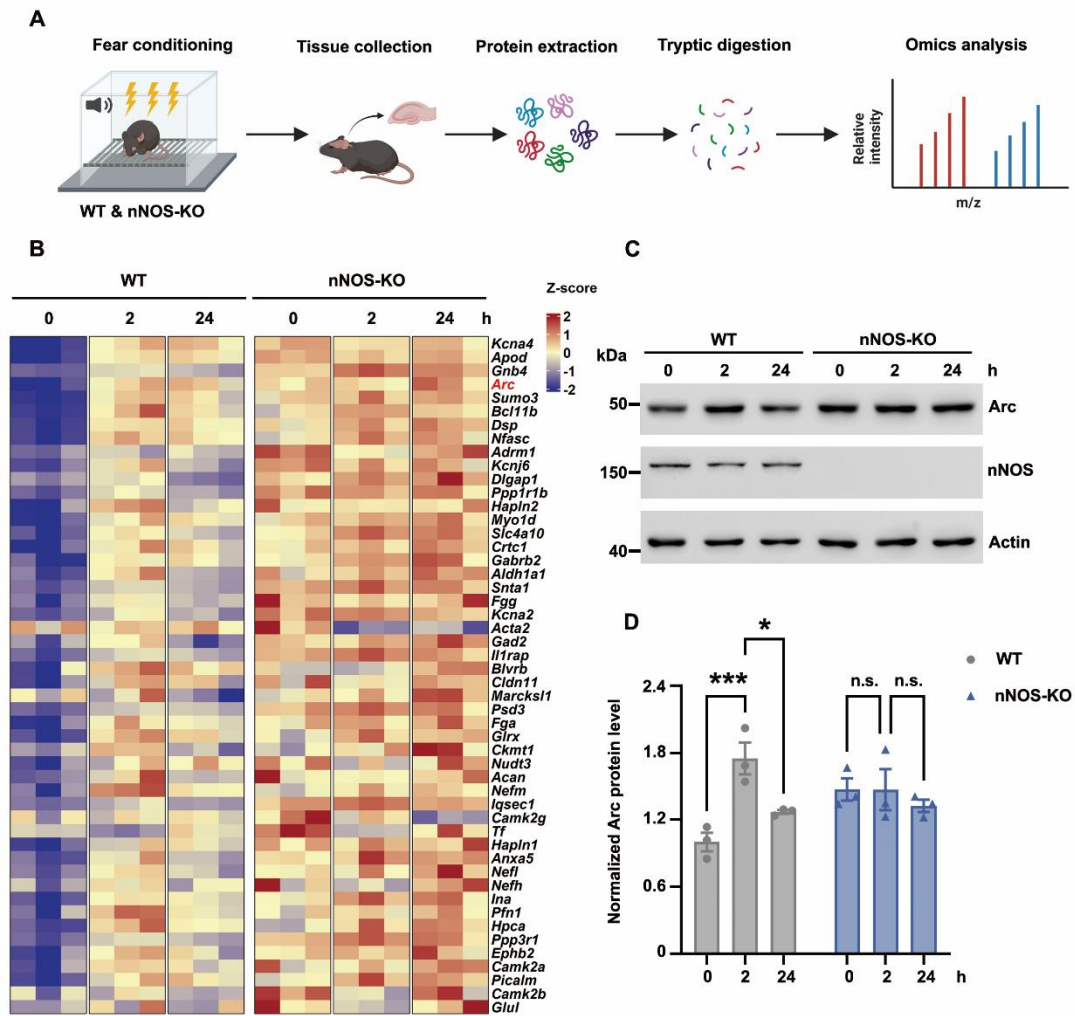


Fig.4 NO promotes the degradation of Arc protein

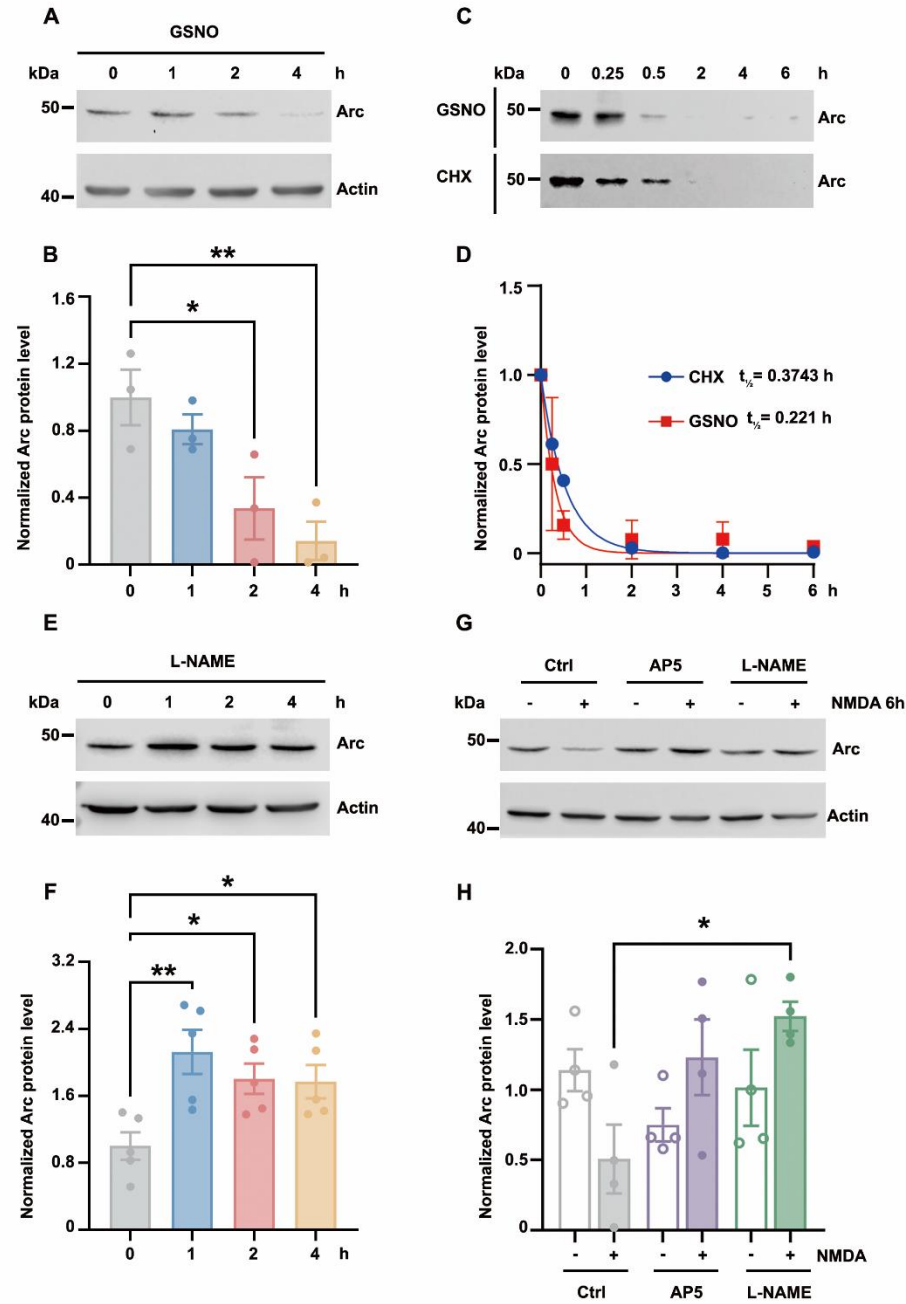


Fig.5 NO S-Nitrosylates Arc at Cys96&159

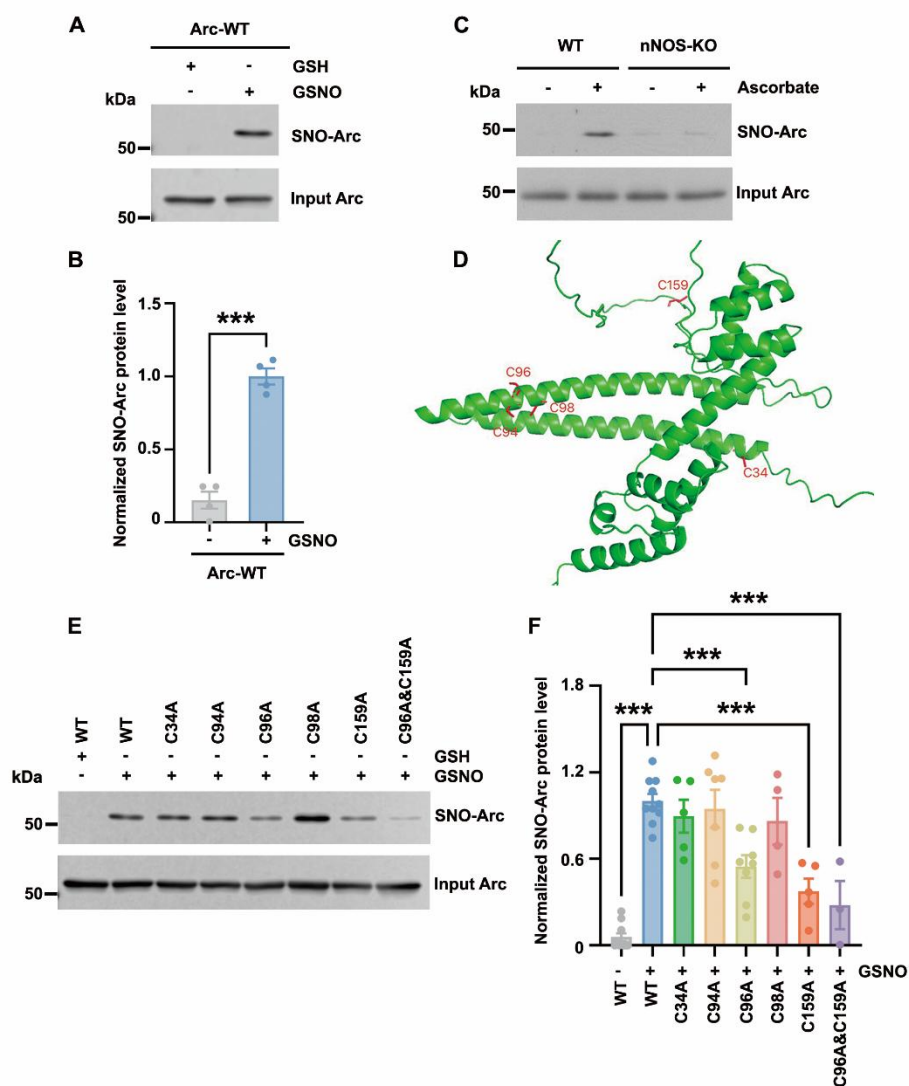
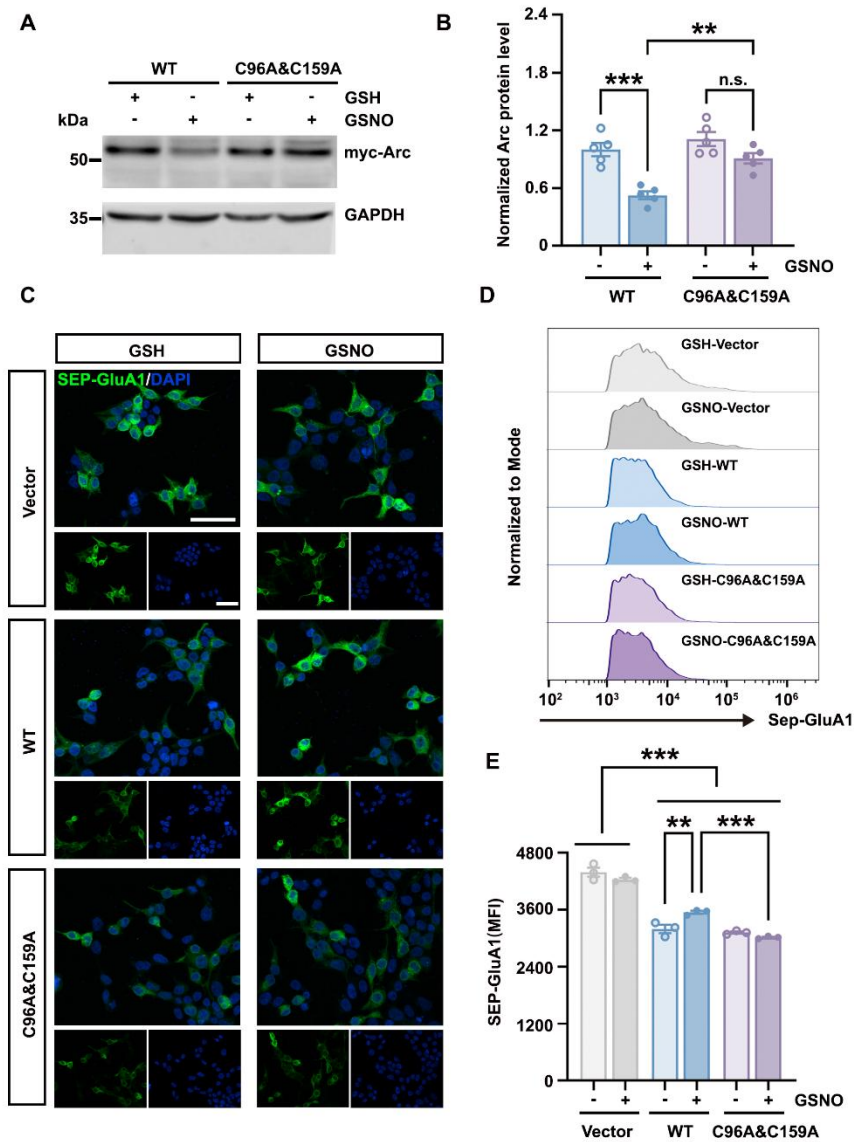


Fig.6 Arc S-Nitrosylation at Cys96&159 mediates NO-dependent regulation of surface AMPA receptor availability



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