

## Perspective

# Nitric oxide-mediated epigenetic mechanisms in developing neurons

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Epigenetic changes of chromatin are increasingly recognized as key modifications that dictate the differentiation state of cells during development. Within the central nervous system, extracellular cues induce chromatin remodelling events that are essential for neuronal progenitor proliferation, cell differentiation and, later, plasticity. In this review, we discuss recent studies that show how extracellular and intranuclear signals influence chromatin remodeling and neuron-specific gene expression. The gaseous molecule Nitric Oxide (NO) has recently emerged as a new key player that mediates the epigenetic changes associated with cell cycle arrest and differentiation in neurons. Histone deacetylases (HDACs) are the first identified intranuclear targets of NO, but, due to its highly diffusible nature, it is likely that many other nuclear factors are directly regulated by NO.

## Introduction

The development of the nervous system requires the generation of a large number of neuronal cell types from a single population of progenitor cells. Initial cell fate determination of neuronal precursors is followed by neuronal migration to target destinations, axonal elongation and establishment of contacts with specific target cells. All these events are associated with a tightly regulated temporal and spatial expression of transcription factors, which is mediated by both extrinsic signals, including Notch, ephrins and neurotrophins, and intrinsic factors, such as chromatin remodeling factors.<sup>1-3</sup> It is now recognized that epigenetic modifications of DNA and histone proteins influence the overall structure of chromatin, regulating the access of transcription factors to gene promoters and driving transcription.<sup>4-7</sup>

Adrian Bird has recently proposed a revised definition of epigenetics as “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states”.<sup>8</sup> DNA is packaged into chromatin through its association with protein complexes called nucleosomes. A nucleosomal unit comprises 146 base pairs of DNA wrapped around an octameric histone complex composed of one H3/H4 tetramer and two H2A/H2B dimers.<sup>9</sup> Nucleosomes are

connected by 20–60 base pairs of DNA associated with the linker histone H1. Chromatin exists either as highly condensed heterochromatin or as the less compact euchromatin. Heterochromatin is late-replicating, rich in repetitive sequences, and transcriptionally silent, whereas euchromatin organisation is more dynamic and can change transiently, allowing for gene activation and repression in response to extracellular cues.

Epigenetic regulation of chromatin involves covalent modification of both DNA and histone proteins.<sup>4,7</sup> Histones can undergo a large number of posttranslational covalent modifications at their N-terminal tails, including acetylation, methylation, phosphorylation, ADP-ribosylation, sumoylation and ubiquitination.<sup>4-7</sup> Histone acetylation, the most widely studied epigenetic modification, involves the cleavage of acetyl groups from acetyl-coenzyme A and its addition to the  $\epsilon$ -NH<sup>+</sup> of lysine residues. Acetylation of histones is catalysed by histone acetyl-transferases (HATs), which are divided into three main families, CBP/p300, MYST and GNAT and exhibit virtually no preference for specific lysine residues.<sup>10,11</sup> Histone acetylation directly influences chromatin structure by reducing the positive charge on lysine residues and decreasing the affinity of histones for negatively charged DNA. This allows a localized ‘unravelling’ of chromatin, making it more accessible for binding of further co-activators and the basal transcription machinery. Histone acetylation also provides a platform for chromatin remodelling proteins that contain bromo-domains, such as the SWI/SNF complex.<sup>7,12</sup> The assembly of these large multiprotein complexes is important for the initiation of transcription, DNA replication and repair and further histone modifications.

Conversely, the removal of acetyl groups is catalysed by histone deacetylases (HDACs) and is associated with chromatin condensing and transcriptional repression.<sup>13-18</sup> There are four classes of HDACs, class I (HDACs 1–3 and 8), class II (HDACs 4–7, 9 and 10) and class IV (HDAC 11) that are all zinc dependent, whereas class III HDACs (SIRT 1–7) require the cofactor NAD<sup>+</sup>.<sup>16,19</sup> Class I HDACs show high homology to the yeast Reduced potassium dependency-3 (Rpd3) and, with the exception of HDAC3, are ubiquitously expressed and principally localized in the nucleus. Class II HDACs are homologous to yeast Histone deacetylase-1 (Hda-1). They exhibit a tissue-specific expression and shuttle between the nucleus and cytoplasm (as does HDAC3). HDAC11, the sole member of class IV HDACs identified so far, shows an intermediate homology to both Rpd3 and Hda1. Sirtuins (class III HDACs), are homologous to silent information regulator-2 (sir2) and are evolutionarily distinct from classes I, II and IV HDACs.

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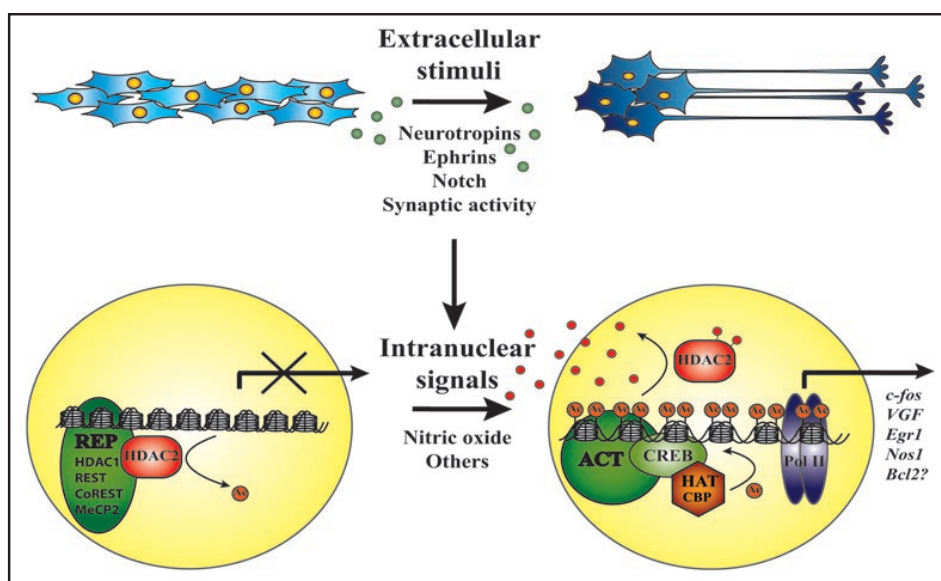


Figure 1. Neuronal progenitor cells exist in a proliferative state, and terminally differentiate to postmitotic neurons upon exposure to extrinsic stimuli, such as neurotrophins, ephrins, notch signaling and synaptic activity (upper). In proliferating neuronal precursors, HDAC2 is recruited to neural gene promoters through interaction with repressive complexes (REP), which include the corepressors HDAC1, REST, CoREST and MeCP2. Recruitment of HDAC2 to chromatin maintains local histones in a deacetylated state and is associated with transcriptional repression. Following extracellular stimulation NO accumulates within the nucleus, and S-nitrosylated HDAC2 is released from repressive complexes (lower). Dissociation of HDAC2 from neural gene promoters, together with the recruitment of transcription factors and coactivators, such as CREB and CBP, induces histone acetylation and a shift of chromatin to an active state. This event initiates the expression of many neuronal genes, including *c-fos*, *VGF*, *Egr1*, *Nos1* and *Bcl2*.

## Epigenetic Mechanisms in the Developing Nervous System

The expression of neural genes during development requires the turnover of large transcriptional complexes that often contain either HATs or HDACs.<sup>20</sup> During the early stages of neuronal development, progenitor cells are maintained in a proliferative state through expression of Notch effectors. These include inhibitory basic helix-loop-helix (bHLH) transcription factors, such as *Hes1*, that act in concert with co-repressive complexes to inhibit neurogenic bHLH genes, such as *Ascl1*.<sup>21</sup> The repressive activity of *Hes1* is mediated by the recruitment of Sin3A/HDAC complexes to gene promoters that results in localized histone hypoacetylation and gene silencing.<sup>22</sup> Exposure of progenitor cells to neurogenic stimuli leads to ADP-ribosylation of the *Hes1* complex, which induces the dissociation of Sin3A/HDAC complex from *Hes1*, recruitment of HATs and hyperacetylation of local histone proteins.<sup>23</sup> The switch of *Hes1* from a transcriptional repressor to an activator is dependent on the presence of HDACs and HATs within the complex, suggesting that the conventional distinction between nuclear repressors and activators is becoming obsolete. The nuclear factor CREST for example, was initially described as a calcium-responsive transcriptional activator that regulates activity-dependent dendritic development.<sup>24</sup> Recent studies however, have shown that CREST is tightly associated with the nBAF chromatin remodelling complexes, and can either activate or repress transcription by recruiting specific cofactors to gene promoters.<sup>25</sup> In resting neurons, CREST interacts with the nBAF subunit Brg1 and represses transcription by recruiting HDAC1 and Rb complexes.<sup>26</sup> Following exposure of cortical culture to depolarizing conditions, Rb and HDAC1 are released from gene promoters and the CREST/Brg1 complexes associate with CBP, inducing histone hyperacetylation and gene expression.

Many neuronal genes contain a conserved motif within their promoters called the Repressor Element 1 (RE1) that upon methylation binds the zinc-finger transcriptional repressor RE1 silencing transcription factor (REST).<sup>27,28</sup> REST recruits Sin3A/HDAC, as well as the repressive cofactors CoREST and MeCP2, to sites of gene repression. CoREST is a co-repressor that mediates long-term gene silencing by recruitment of HDACs, H3K9 histone methyltransferases and the H3K4 demethylase LSD1.<sup>29,30</sup> REST silences many neuron-specific genes in non-neuronal cells, including those encoding ion channels, neurotransmitter receptors, synaptic vesicle proteins and adhesion proteins.<sup>31</sup> Transcriptional and translational regulation of REST allows a tight control of neuron-specific genes during development, and later influences the plasticity of mature neurons.<sup>32</sup> High levels of REST in neuronal embryonic stem cells prevent their exit from the cell cycle and blocks premature neuronal differentiation.<sup>33</sup> As neuronal development proceeds, the *Rest* gene itself is transcriptionally and translationally repressed, allowing precursor cells to differentiate into mature neurons.<sup>33</sup> The absence of REST/HDAC/Sin3A/CoREST repressive complexes from gene promoters induces the expression of neuronal genes.<sup>34</sup> However, the transcription of certain genes, including *Bdnf* and calbindin, remains low due to CoREST and MeCP2 complex binding to additional methylation sites.<sup>33</sup> Following neuronal depolarisation, MeCP2 is phosphorylated, possibly decreasing its binding affinity for methylated DNA and *Bdnf* transcription is induced.<sup>35</sup> Regulation of the binding of MeCP2 repressive complexes to neural gene promoters in response to neuronal activity provides a platform for synaptic plasticity in mature neurons.<sup>32</sup>

Differentiation of progenitor cells into a neuronal phenotype is also regulated by the nuclear receptor co-repressor (N-CoR).<sup>36,37</sup>

Repressive complexes containing N-CoR silence gene promoters of down-stream effectors of Notch signaling through the recruitment of HDAC3.<sup>36</sup> The absence of N-CoR in cortical progenitors prevents FGF-dependent self-renewal of neuronal progenitors and promotes their differentiation into astrocytes.<sup>38</sup> Interestingly, Ciliary Neurotrophic Factor (CNTF) triggers glial differentiation and leads to Akt1 kinase-dependent phosphorylation of N-CoR and subsequent relocalization to the cytoplasm.<sup>38</sup> These data imply that N-CoR/HDAC3 complexes play an important role in regulating the differentiation of early-stage progenitor cells by selectively repressing gliogenic genes.

### Nitric Oxide Regulates Neurogenesis and Neuronal Development

Nitric Oxide (NO) is an important mediator of two pivotal neuronal developmental processes, proliferation and differentiation. The primary source of NO in neurons is the neuronal isoform of nitric oxide synthase (nNOS). After the discovery that NO acts as a physiological signaling molecule, it became apparent that cysteine thiol groups can couple NO to form *S*-nitrosylated proteins, a modification akin to phosphorylation.<sup>39</sup> *S*-nitrosylation has been reported to regulate the function of many proteins, affecting catalytic activity, protein-protein interaction and/or subcellular localisation.<sup>40-42</sup> Anti-proliferative effects of NO have been demonstrated in cerebellar granule cells, as well as in several neuronal-derived cell lines, where NO induces cell cycle arrest without affecting cell viability or apoptosis.<sup>43-46</sup> The observation that NO triggers growth arrest in PC12 cells provided the initial indication that NO is an important regulator of neuronal development.<sup>43,47</sup> Similarly, reduced proliferation and increased differentiation was observed in neuroblastoma cell lines and in cerebellar neurons upon exposure to endogenous and exogenous sources of NO.<sup>44,45,48</sup> Interestingly, stimulation of mouse cortical neuroepithelial cluster cultures with either NO donors or BDNF inhibits cell proliferation and promotes differentiation, possibly by increasing nNOS levels, thereby providing a positive feedback loop.<sup>48</sup> Experiments performed *in vitro* by using embryonic brain slice cultures, or in postnatal rats *in vivo* have shown that exposure to NOS inhibitors impairs cerebellar granule cell migration and increases proliferation.<sup>49,50</sup>

In the adult brain, neurogenesis persists within two regions, the sub ventricular zone (SVZ) and the subgranular zone of the dentate gyrus in the hippocampus. Discrete populations of neurons expressing nNOS are found in close proximity to these highly proliferative regions.<sup>51,52</sup> Neuroblasts from the SVZ migrate tangentially along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate to form mature interneurons. NOS positive cells are found along the RMS, with abundant processes that intermingle with the migrating precursor cells.<sup>51</sup> Interestingly, NOS-negative progenitors in the adult SVZ begin to express NOS as they start migrating to the olfactory bulb, implicating that NO may play a role in regulating this process. Genetic deletion of nNOS, as well as systemic and intraventricular infusion of nNOS inhibitors in adult mice, augmented the number of cells generated within the SVZ and the dentate gyrus.<sup>53-55</sup> Primary cultures of the SVZ obtained from nNOS null mice have also demonstrated an inhibitory effect of NO on proliferation without influencing cell survival.<sup>54,56</sup>

In the developing nervous system, nNOS is transiently expressed in restricted regions of the brain, including the embryonic cortical plate, and the olfactory epithelium.<sup>57</sup> Within these populations, nNOS is not detectable during the early phases of neurogenesis, when neural stem cells are actively proliferating. nNOS mRNA is first detected in neural precursor cells at E10, with levels progressively increasing as precursors later differentiate to form post-mitotic mature neurons.<sup>48,57,58</sup> Neurotrophin-dependent precursors in sensory and sympathetic ganglia also express high levels of nNOS, as they exit the cell cycle and start to differentiate.<sup>57,59</sup> However, nNOS expression dramatically decreases postnatally, once peripheral neuron axons have established connections with their targets.<sup>59</sup> Importantly, stimulation of cortical neurons with the neurotrophic factor BDNF increases expression of nNOS and induces NO accumulation within both cytoplasmic and nuclear compartments.<sup>48,60,61</sup>

### Transcriptional Regulatory Targets of NO

Several studies have demonstrated that NO modulates gene expression through the activation of multiple transcription factors including CREB, N-Myc, NFκB, p53 and in bacteria, OxyR.<sup>62-65</sup> A role of NO in regulating gene expression was first demonstrated in the neuronal-derived cell line, PC12. Structure to function analysis of the *c-fos* promoter demonstrated that Ca<sup>2+</sup> signaling induced NO synthesis and CREB-dependent *c-fos* expression.<sup>66</sup> Other studies have shown that NO activation of CREB regulates neuronal survival, differentiation and plasticity.<sup>60,67-70</sup> However, the mechanisms by which NO regulates CREB activation remain unclear. In cerebellar granule cells, NO-dependent phosphorylation of CREB through the sGC/PKG pathway has been shown to be required for CREB activation and for promoting neuronal survival.<sup>69,71</sup> In contrast, NO produced following neurotrophin stimulation of cortical neurons mediates CREB activity through mechanisms that are completely independent of CREB phosphorylation and involve the recruitment of active CREB to target gene promoters.<sup>60</sup> More importantly, no changes of CREB phosphorylation were observed in cortical neurons obtained from nNOS null mice and exposed to either BDNF or depolarizing stimuli.<sup>60</sup>

An important target of NO is the oncogene N-Myc, a transcription factor that is essential for the expansion of neuronal precursors during normal brain development.<sup>72</sup> NO signaling inhibits N-Myc expression through the cGMP/PKG pathway, decreasing proliferation rates of neonatal precursor cerebellar granule neurons, neuroblastoma cell lines and embryonic carcinoma stem cells.<sup>45,50,73</sup> Despite the many evidences that NO regulates the transcriptional activity of CREB and n-Myc, they are not target of *S*-nitrosylation, and the molecular mechanisms that regulate their activation remain unclear.

*S*-nitrosylation, however, mediates NO-dependent regulation of various zinc-finger-containing transcription factors, including *egr-1* and NFκB.<sup>74,75</sup> *S*-nitrosylation of zinc-sulphur clusters of *egr-1* and NFκB disrupts zinc binding, inhibiting their association with DNA.<sup>76-78</sup> In mammalian cells, NFκB is constitutively expressed and is retained within the cytoplasm by its inhibitory cofactor IκB. Upon IκB phosphorylation and subsequent proteolysis, NFκB is free to translocate to the nucleus to bind target gene promoters.<sup>64</sup> *S*-nitrosylation and tyrosine nitration of NFκB inhibits its binding to DNA.<sup>79,80</sup> Furthermore, *S*-nitrosylation of IκB prevents its phosphorylation thereby increasing cytoplasmic retention of NFκB.<sup>81,82</sup>

Other studies however, have suggested that NO signaling induces the expression of NF $\kappa$ B-regulated genes.<sup>83,84</sup> Similar conflicting evidences have been observed for the transcription factor AP1. NO has been demonstrated to inhibit AP1 binding to DNA, through modification of its two components, *c-fos* and *c-jun*.<sup>85,86</sup> In contrast, NO signaling induces expression of the *c-fos*-dependent gene, tyrosine hydroxylase, as well as *c-fos* itself, in a number of neuronal systems.<sup>60,87-89</sup> This may serve as a negative feedback mechanism, as NO initially induces expression of *c-fos* and later inhibits AP1 activity through *S*-nitrosylation of *c-fos* and *c-jun*.

A common link between the physiological processes regulated by NO during the development of the nervous system is the requirement for profound and long-lasting changes of gene expression. Exposure of cells to NO donors induces the transcription of a large number of genes through the binding of transcription factors to various cis-activating elements, including CRE, AP1, AP2, BRN-3, EGR, E2F1 and Sp1.<sup>90,91</sup> NO regulates a broad range of functionally distinct genes involved in cell signaling, cell cycle, transcription, stress response, protein degradation, oxidative stress and apoptosis.<sup>90</sup> The large number of transcription factors activated and the diversity of genes induced suggest that NO is likely to signal at an epigenetic level, reflecting the broad impact that NO has on neuronal physiology.

### Regulation of HDAC Function by NO Signaling

Neurotrophin stimulation and NO signaling are important modulators of cortical neurogenesis. Both BDNF and NO inhibit proliferation and promote neuronal differentiation.<sup>48,53,55,92,93</sup> BDNF stimulation of cortical neurons induces accumulation of NO within the cytoplasmic and nuclear compartments.<sup>61</sup> Furthermore, acetylation of histones H3 and H4 increases following BDNF treatment of cortical neurons in a NO-dependent manner.<sup>60,61</sup> Recently, the chromatin-modifying enzyme HDAC2 has been identified as a key nuclear target of NO.<sup>61,94</sup> *S*-nitrosylation of HDAC2 occurs at two cysteine residues, Cys262 and Cys274, and does not affect its deacetylase activity in neurons. Instead, HDAC2 *S*-nitrosylation induces its dissociation from CREB-regulated gene promoters. This event leads to an increase of histone acetylation at specific promoter regions and transcription of many genes, including *c-fos*, *egr1*, *VGF* and *nNos*.<sup>60,61</sup> Importantly, NO-dependent modulation of HDAC2 function is necessary for dendritic growth *in vitro*, suggesting a key role of HDAC2 *S*-nitrosylation in cortical development.<sup>61</sup>

NO-dependent inhibition of HDAC2 function has also been reported in muscle cells.<sup>94</sup> In a mouse model of muscular dystrophy, NO-dependent inhibition of HDAC2 has been exploited as a potential novel therapeutic approach for this genetic disease.<sup>94</sup> Interestingly, in muscle cells, *S*-nitrosylation of HDAC2 on yet unidentified cysteine residues decreases its deacetylase activity,<sup>94</sup> although in neurons HDAC2 enzymatic activity is unchanged.<sup>61</sup> This discrepancy may reflect a difference of cysteine residue(s) targeted by NO in muscle cells and in neurons. For example, in muscle cells HDAC2 *S*-nitrosylation may occur at Cys152, which is located within the deacetylase active site. It should also be considered that the transcriptional profile between muscle cells and neurons differs greatly, possibly involving the recruitment of HDAC2 to repressive complexes of cell-type specific genes through distinct mechanisms. With the exception of HDAC8, the deacetylase activity of HDACs

is dependent on protein-protein interaction with corepressors.<sup>95,96</sup> Similarly, HDACs do not directly bind DNA and relies on the recruitment by corepressors to gene promoters. An attractive hypothesis is that *S*-nitrosylation of HDAC2 results in its dissociation from cell-type specific repressive complexes,<sup>26</sup> which may influence its association with chromatin and/or deacetylase activity (Fig. 1).

Regulation of deacetylase function by NO is not restricted to class I HDACs. In endothelial cells, a shear-stress response enhances NO synthesis and inhibits gene expression.<sup>97,98</sup> Treatment of Human Umbilical Vein Endothelial Cells (HUVECs) with NO donors induced nuclear localisation of HDAC4 and HDAC5 and class II HDAC-dependent hypoacetylation of histone H3.<sup>99</sup> Nuclear localisation of class II HDACs is inhibited following phosphorylation by calcium-calmodulin dependent kinases (CaMKs) and its association with the cytoplasmic chaperone, 14-3-3.<sup>100,101</sup> Exposure of HDAC4 to NO donors increases both its association with the protein phosphatase PP2A<sup>99</sup> and the phosphatase activity of PP2A, allowing HDAC4 to translocate to the nucleus. Interestingly, in neurons, NO inhibits deacetylase activity of cytoplasmic HDACs, although nuclear HDAC activity remains unaffected.<sup>102</sup> These results imply that NO signaling in neuronal cells specifically affects the enzymatic activity of cytoplasmic class II HDACs, possibly influencing deacetylation of non-histone proteins.

### Conclusions

The finding that NO regulates many aspects of gene expression in mammalian neurons has implications that reach beyond the regulation of cortical development, and include novel therapeutic approaches for both neurodegenerative disorders and cancer. Regulation of histone acetylation has been implicated in the pathogenesis of several neurodegenerative disorders, including Huntington's, Parkinson's and Alzheimer's diseases.<sup>103-105</sup> These neurodegenerative disorders are associated with the formation of cytoplasmic aggregates that sequester intracellular proteins, interfering with various signaling pathways.<sup>106,107</sup> It is now apparent that inclusion of transcriptional cofactors within these aggregates profoundly affects histone modification and gene expression.<sup>104,108</sup> Interestingly, dysregulation of HDAC2 binding to neuronal gene promoters further exacerbates the transcriptional changes observed in neurons obtained from a mouse model of Huntington's disease.<sup>109,110</sup>

As HDACs play an important role in regulating cell proliferation, differentiation and apoptosis, it is not surprising that they have been a major focus for cancer therapeutics for the past 10 years.<sup>111-113</sup> HDAC2 is highly expressed in gastric carcinomas, colorectal carcinomas, cervical dysplasias and endometrial stromal sarcomas.<sup>114-116</sup> Dysregulated expression of HDAC2 in cancers is associated with reduced transcription of antiproliferative genes such as the cyclin-dependent kinase inhibitor p21.<sup>117,118</sup> NO-dependent regulation of HDAC2 binding affinity for DNA provides an alternative avenue for future drug design that does not require inhibition of intrinsic deacetylase activity. Novel therapeutic approaches may target the chromatin-remodelling function of HDAC2, leaving deacetylation of non-chromatin associated factors intact, thereby providing an avenue for the development of more specific drugs.

The finding that a gaseous molecule, such as NO, has such a profound effect on the transcriptional profile of mammalian, yeast and bacterial cells,<sup>62,76,90,91,119</sup> by directly modifying a large number

of nuclear proteins,<sup>61</sup> suggest that NO may act as a master regulator of gene expression. Future research will reveal to which extent chromatin remodelling factors and nuclear events are functionally regulated by NO.

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