**Functional Characterization of Basic Helix-Loop-Helix-PAS Type Transcription Factor NXF in Vivo**

**PUTATIVE INVOLVEMENT IN AN “ON DEMAND” NEUROPROTECTION SYSTEM**

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NXF, a member of the basic helix-loop-helix-PAS transcription factor family, is thought to be involved in functional regulation of neurons, because significant expression is found in the mature brain. To elucidate functions of NXF in vivo, here we generated mice lacking NXF using homologous recombination with embryonic stem cells. NXF<sup>−/−</sup> mice were morphologically indistinguishable (with no growth retardation) from their littermates (wild type) at birth. However, they started to die at a rate of 1 death/20–30 animals per week under specific pathogen-free grade breeding conditions when over 3 months old. Histological analyses revealed age-dependent neurodegeneration in brain, and only 20–30% of the NXF<sup>−/−</sup> mice survived for 16 months. To clarify the role of NXF in protection against neurodegeneration in normal cells, we analyzed gene expression under several conditions *in vitro* and *in vivo*. The NXF gene was up-regulated by several neurodegenerative cell-stress inducers such as thapsigargin (endoplasmic reticulum stress), SIN-1 (oxidative stress), and sorbitol (osmotic stress) in cultured cells. Furthermore, elevated NXF gene expression was apparent with *in vivo* stroke models featuring kainate-induced hippocampal injury and transient global ischemia. When NXF<sup>−/−</sup> mice were evaluated in the glutamate excitotoxicity model, they proved more susceptible to hippocampal injury at 15 weeks after birth. The findings in this study suggest that the NXF gene could be induced in response to several neurodegenerative stimuli/excitations for the cell protection, and thus provide an "on demand" cell-protection system in nervous tissue.

The basic helix-loop-helix-PAS type protein family is an important class of transcription factors that are generally responsive to various environmental and physiological, including developmental, stimuli and that play roles in cell adaptation as "sensor" proteins, regulating target gene expression (1). Family members have been identified from insects to humans, and *Drosophila* Sim, for example, has been well characterized as a master regulator that is critical for *Drosophila* midline development (2). At least two homologues of Sim (Sim1 and Sim2) are known to be present in the human genome (3), and one of them, Sim2, has attracted interest in the context of Down syndrome pathogenesis (4–6). In the course of a search for additional hypothetical human Sim homologues (hypothetical Sim3), we previously isolated a novel gene, “human NXF” (7), whose C-terminal half demonstrated no homology to all known other members of the basic helix-loop-helix-PAS family (including Sim1/2). *Drosophila* and *Caenorhabditis elegans* also have NXF-like genes, and the *Drosophila* example (called Dys (8)) has been functionally characterized by gene knock-down. The expression pattern of the *Drosophila* NXF-like gene exhibits little tissue specificity, unlike the mammalian NXF gene that is selectively expressed in the nervous system. Although the *Drosophila* NXF-like gene has been shown to be critical for development of the *Drosophila* trachea (8), mammalian NXF is only slightly expressed in the mammalian development stage, rather becoming evident when development of all tissues is complete (7). The observed clear increase in expression in mammalian NXF in the nervous system after birth suggests important roles in neural functions in the mature brain.

In the present report, we document “on demand” expression of NXF in cultured cells treated with several cell-stress inducers *in vitro*, and in two animal models accompanying neurodegeneration *in vivo*. To investigate the significance of NXF-inducible expression *in vivo*, we utilized NXF-deficient (null: NXF<sup>−/−</sup>) mice. NXF<sup>−/−</sup> mice were evaluated in the kainate-induced hippocampal injury model (excitotoxicity model). Comparison of the NXF<sup>−/−</sup> mouse with the wild-type littermate provides insights into important NXF function *in vivo*.

**EXPERIMENTAL PROCEDURES**

**Targeted Disruption of NXF**—A targeting vector was constructed using a neo gene cassette for positive selection and a diphtheria toxin A-chain gene cassette for negative selection. NXF exons 1–3 (2.1-kb EcoRI-EcoRV genomic fragment) was replaced by the neo gene cassette. neo was flanked by 4.3-kb (EcoRV-Scal) and 4.7-kb (HpaI-EcoRI) NXF genomic sequences derived from the 129/Sv genomic library. The diphtheria toxin A-chain gene cassette was ligated to the Hpal site. After linearization, this construct was electroporated into 129/Sv-derived embryonic stem (ES)<sup>2</sup> cells (Xenogen Biosciences Corp.), and G418-resistant colonies were selected. Homologous recombination events were assessed and confirmed by Southern blot hybridization. Two independent clones (the targeted ES clones) were injected into C57BL/6 blastocysts and transferred to foster mothers to obtain chimeric mice. Chi-

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² The abbreviations used are: ES, embryonic stem; WT, wild type; KO, knock-out; GFAP, glial fibrillary acidic protein.
NXF KO Mice

morphic males were then mated with C57BL/6 females and F1 heterozygous mice carrying the NXF mutation were identified by Southern blot hybridization and intercrossed to produce F2 homozygous offspring. Two independent lines of F4 mice were assessed in the present study. No essential differences were observed between these two lines of NXF-deficient mice, and also between F4 and F8 generations examined later for confirmation. All wild-type (WT) mice used in each assay were wild-type littermates of the NXF-deficient mice analyzed.

Experimental Animal Treatment—Experiments using rats and mice were performed in accordance with the Institutional Animal Care and Use Committee guidelines. 15-week-old C57BL/6 (Charles River, Tokyo, Japan) mice, NXF-deficient mice, and wild-type littermates were injected (intraperitoneally) with saline (vehicle) or 28 mg/kg sodium kainate (kainate, Wako, Japan) and the brains were removed, fixed in 4% glutaraldehyde/phosphate-buffered saline, and embedded in paraffin 5 days thereafter for immunostaining. Transient global ischemia treatment was performed in Nihon Bioresearch Center (Gifu, Japan). For this purpose, 8-week-old Crj:Wistar rats (Charles River, Tokyo, Japan) were anesthetized, and the vertebral arteries were closed by heat-coagulation at the first cervical vertebrae. On the day after this irreversible obliteration, both common carotids exposed in the cervical region were temporarily closed for 10 min with clips (Sugita Clip, Hozumikakogo, Japan). At 10 days after the transient ischemia, the brains were fixed, paraffin-embedded, and subjected to immunostaining.

Cell Culture and Stress Treatments—PC12 cells (ATCC) cultured in RPMI1640 supplemented with 5% fetal calf serum, 15% horse serum and 1 mM sodium pyruvate for 16 h, then stimulated with medium containing vehicle (final 0.1% DMSO), 10 μM SIN-1 (Dojin Chemical, Japan) plus 1 mg/ml bovine serum albumin or 1 mg (6160 units)/ml SOD (Nakarai, Japan), 1 μM thapsigargin (Wako, Japan), 1 μM SIN-1 (Dojin Chemical, Japan) plus 1 mg/ml bovine serum albumin or 1 mg (6160 units)/ml SOD (Nakaritesuku, Japan), or 1 mM sorbitol (Wako, Japan). Cells were harvested at the indicated time points, and extracted RNAs were subjected to real-time PCR analysis with a rat NXF-specific TaqMan probe (Rn00596522_ml; ABI). The level of NXF (n = 6) was normalized with a control TaqMan probe (glyceraldehyde-3-phosphate dehydrogenase, ABI).

Whole Brain Real-time PCR—NXF-deficient mice (KO) and wild-type littermates (WT) were sacrificed at 15 weeks (n = 10 each) or 30 weeks (n = 10 for each) of age, and RNAs from half of whole brain (right hemisphere) were subjected to real-time PCR analysis with mouse NXF-specific TaqMan probes (Mm00463644_ml, ABI), normalized to a control TaqMan probe (glyceraldehyde-3-phosphate dehydrogenase, ABI). Similarly, TaqMan probes for GAP (Mm00546086_ml, ABI), XBP-1 (Mm00457359_ml, ABI), GRP78 (Mm00517691_ml, ABI), ATF6 (Mm01295316_ml, ABI), Ern1 (Mm00470233_ml, ABI), GPX1 (Mm00656767_ml, ABI), and GPX2 (Mm00850074_gl, ABI) were used for quantification of the respective gene expression levels.

Histopathological Analyses—Fluoro-jade B (Chemicon), an anionic fluorescein derivative that has specific affinity for neurons undergoing degeneration, was used to stain animal brain sections following a standard procedure (9). Deparaffined sections were treated in 0.06% KMnO4 solution for 15 min and then stained with 0.001% Fluoro-jade B/0.1% acetic acid solution for 30 min. The tissue was then examined under a fluorescence microscope with the filter set for fluorescein isothiocyanate to visualize Fluoro-jade B fluorescence (Olympus, IX-FLA-DP50 system). For immunostaining, tissue sections (10-μm thickness) from paraffin-embedded brains were activated (100 °C 5min) in Target Retrieval Solution (DAKO), then exposed to anti-NXF, anti-plunK (SC-6254, Santa Cruz Biotechnology), anti-Iba1 (Wako), or anti-Nitrotyrosine (AB5411, Chemicon) antibodies. For production of anti-NXF antibodies, rabbits were immunized with a keyhole limpet hemocyanin-conjugated peptide (CRFNTSKSLRRQSAKKNL) specific for NXF, using a standard immunization protocol (Asahi Tekuno Glass, Chiba, Japan). The IgG fraction from pooled antisera was purified using a standard affinity method for use in immunostaining. Visualization of binding was performed with a 3,3′-diaminobenzidine tetrahydrochloride staining kit (Histofine simple stain MxA-PO kit and 3,3′-diaminobenzidinetetrahydrochloride solution, Nichirei, Tokyo, Japan). For anti-GFAP immunostaining, a commercially available GFAP staining kit containing anti-GFAP antibody was employed (Histofine SAB-PO GFAP kit, Nichirei). A terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) kit (ApopTag Plus Peroxidase In situ Apoptosis Detection Kit, Chemicon) was used for detection of apoptosis by the TUNEL method.

RESULTS AND DISCUSSION

Generation of NXF-deficient (Null) Mice—A targeting vector for the mouse NXF gene was created by inserting a neomycin resistance cassette into the NXF locus genomic fragment derived from the 129/Sv mouse, replacing the promoter and exons 1–3, which encode the DNA binding domain essential for NXF function (Fig. 1A). The targeting vector was introduced into ES cells through homologous recombination, and proper recombination events were identified using Southern blot analysis. Several independent recombinant ES clones were obtained and microinjected into C57BL/6 mouse blastocysts to generate chimeric mice. Several lines of chimeric mice derived from independent mutant ES clones were found to transmit the mutant allele to their offspring. Through animal breeding, wild-type, homozygous, and heterozygous mutant genotypes were generated and confirmed by Southern blot analysis of DNA from the progeny obtained (Fig. 1B), and two independent lines of NXF-deficient mice derived from two independent recombinant ES clones were obtained. Such homologous mutation of the NXF gene should lead to NXF functional deficiency (NXF−/−) in the mice, because it results in no promoter and no DNA binding domain. To confirm the lack of expression of NXF, we performed real-time PCR analysis using total RNAs of brains from wild-type and homozygous mice. As with null mice, no significant expression of NXF was detected in both lines of NXF-deficient (null) mice (Fig. 1C, KO-(1) and KO-(2)).

Progressive Brain Damage Observed in NXF-deficient Mice—Newborn NXF-deficient mice showed no morphological abnormality, compared with their littermates (wild type) at
...direct target gene products, which remain to be identified.

**FIGURE 1.** Generation of NXF-deficient (null) mice by gene targeting. A, schematic representation of the NXF genomic structure, the targeting construct, the targeted alleles of NXF, and the Southern blot analysis strategy used to confirm the mutated locus structure. B, Southern blot of SpfI-digested genomic DNA from a candidate targeted mouse. The wild-type (WT) allele from a wild-type littermate is 8.9 kb (lane 3), and the targeted allele from an NXF-deficient (NXF"--") mouse is 6.4 kb (lane 4). The blots were probed with the 5’ probe shown in panel A. Lane 1 is the control in which total genomic DNA from the originally identified chimera mouse was analyzed in parallel. Lane 2 is for the other candidate, which was later unused. C, real-time PCR analysis of RNAs from WT and NXF-deficient (KO) whole brain tissues with the TaqMan probe specific for mouse NXF (n = 5 animals for each). Both independent lines of NXF-targeted mice (KO(1) and KO(2)) lack detectable (N.D.) NXF mRNA.

birth, and demonstrated the expected Mendelian ratio and bred normally, indicating that NXF is dispensable for embryonic development. The NXF-deficient mice showed no growth retardation and no significant abnormality at young age. However, after ~3 months, a small percent of NXF-deficient mice in both independent NXF-null lines started to show abnormal behavior such as crouching, bending back, or abnormal jumping, often resulting in self-injury, suggesting some neurological abnormality (data not shown). Although their symptoms and severity differed greatly each other, such abnormalities progressively developed and NXF-deficient mice finally start to die at a rate of 1 death/20–30 animals per week under clean (specific pathogen-free grade) breeding conditions. Differences in survival rates among NXF-deficient mice, heterozygotes, and control wild mice (littermates) are illustrated in Fig. 2A. Although almost all wild mice survived 16 months, this was the case for only 20–30% of NXF-deficient mice. In addition, many of the NXF-deficient survivors demonstrated abnormal behavior (mentioned above). The heterozygote (NXF"+-") mice also showed vulnerability compared with control wild-type mice, some 20% dying during the 16 months. In the NXF-deficient mice brains on histopathological analyses, performed when they died or they were dying, severe neuronal cell damage accompanied by heavy glial cell activation was apparent with no abnormality in any of the other major tissues examined.

As a first step in characterizing the brain damage in detail, expression levels of a glial cell activation marker, GFAP, in NXF-deficient mice (KO1–10) and control wild littermates (WT1–10) were examined (Fig. 2B). Induction of GFAP, which indicates glial cell activation, is known to accompany proximal neuronal cell damage, and is often used as an indirect marker of brain damage (10). When 15 weeks old, NXF-deficient (null) mice (KO1–10) in this experiment, lacking significant abnormality in behavior, did not show any significant difference in the GFAP mRNA expression level as compared with control wild-type littermates (WT1–10) (Fig. 2B, upper panel). However, in the NXF-deficient brains at 30 weeks of age, significant induction of GFAP mRNA was detected, albeit with marked variation in the extent (Fig. 2B, lower panel). In this experiment, there were no deaths of NXF-deficient mice (KO11–20) at 30 weeks of age. Histopathological analyses of the NXF-deficient mice brains, however, confirmed GFAP induction in glial cells (data described in the next section). Using the same RNA samples, we also quantified the expression level of several genes related to cell-stress tolerance. Among cell-stress-responsive-/related genes tested (over 50 genes), XBP-1 (11), GRP78 (12), Ern1 (13), and GPX2 (14) declined in their expression in the NXF-deficient mice at 30 weeks of age (Fig. 2C). These may be examples of indirect NXF target genes that are responsible for cell-stress tolerance preventing damage of brain such as that observed in NXF-null mice. XBP-1 and Ern1 function as ER stress sensor proteins, whereas GRP78 and GPX2 are also stress-inducible cell-protective proteins that are important for cell-stress tolerance. It could be anticipated that the NXF-deficient brain at 30 weeks of age is more susceptible to cell stress than at 15 weeks, contributing to progressive brain damage. A spiral toward cell death could clearly originate from the decreased expression of stress-tolerance genes. All of the above genes had normal expression at the 15-week time point. Thus, as another possibility, these genes may be secondarily or thirdly influenced by a certain event that should be regulated by some NXF direct target gene products, which remain to be identified in future investigations. On the other hand, nonspecific gene down-expression or simple cell loss in the brain does not appear to contribute to the repressed gene expression observed at 30
NXF KO Mice

![Progressive brain damage observed in NXF-deficient mice.](image)

A, survival rates of NXF-deficient mice (NXF-/-), heterogenic mice (NXF+/-), and wild-type littermates (Wild). B, expression levels of GFAP, a marker of glial cell activation and proximal neuronal cell damage. Whole brain RNAs of NXF-deficient mice (KO) or wild-type littermates (WT) at 15 weeks (n = 10 for each) or 30 weeks old age (n = 10 for each) were quantified individually for GFAP mRNA (Normalized) / Fold (data not shown). C, several stress-related genes demonstrate decreased expression in NXF-deficient brains (KO). Expression levels of each gene in the brains of NXF-deficient mice (KO) or control wild-type littermates (WT) were quantified by real-time PCR (n = 10 for each) with the respective TaqMan probes (RNA samples were prepared from whole brain).

weeks of age in this experiment, because ATF6 (15) and GPX1 (16) as control genes here and internal control glyceraldehyde-3-phosphate dehydrogenase (and over 50 genes with other factors tested; data not shown) were unaltered.

**Histopathologically Detected Brain Damage in NXF-deficient Mice**—To confirm the brain damage, we performed a histopathological analysis of NXF-deficient brains. No differences between NXF-deficient and wild-type mice (and among 10 animals for each) were evident at 15 weeks of age (data not shown). However, at 30 weeks, obvious degeneration of neurons was evident in the NXF-deficient animals sacrificed for histopathological analyses. Degeneration of neurons was also detected by Fluoro-jade B (known to specifically stain degenerated neurons (9)) fluorescence staining analysis (Fig. 3A). It should be pointed out that there were large individual differences in the damaged brain sites and the degree of injury each other. In some NXF-deficient mouse brains, almost all hippocampal CA1–2 neurons had already died. An example of individual damage differences is shown figure 3B with anti-GFAP immunostaining, a conventional tool to show proximal neuron damage. Individual NXF-deficient mice (KO11–13) showed different distribution patterns of GFAP immunoreactivity in the hippocampus (Fig. 3B Hippocampus), although brain damage was not restricted to the hippocampus (Fig. 3B lower panel: Cerebral cortex). In the NXF-deficient brains, the TUNEL method (17) detected apoptosis (Fig. 3C, top panel). Gathering of microglial cells was also observed (Fig. 3C, middle panel), as often observed in the degenerating brain after ischemia (18). The presence of oxidized proteins with nitrotyrosines (19) suggested that oxidation could be involved in neuronal damage observed in the NXF-deficient mice (Fig. 3C, bottom panel).

The pathology of brain damage was reminiscent of that observed in cerebral stroke (20). Because NXF-deficient mice did not exhibit seizures or epilepsy during daily observation, they do not appear to suffer from stroke, epilepsy, ischemia, or related symptoms, which themselves could lead to neuronal damage. Rather, environmental neural stresses such as autoexcitotoxicity in daily life might be responsible. If so, NXF could be an essential molecule for neural stress responses and neuronal cell survival in the mature brain.

**On Demand NXF Expression in Vitro and in Vivo**—It is well known that, as a kind of self-defense mechanism, several cell-protective genes are induced by cell stresses in parallel with cell damage (21, 22). We therefore examined the effects of several typical neurodegenerative stress stimuli on NXF mRNA expression in PC12 cells. A typical ER-stress inducer, thapsigargin (10 μM) (23), induced NXF mRNA with a peak at 0.5 h (Fig. 4A, top panel). With a typical oxidative stress inducer, SIN-1 (1 mM) (24), the peak occurred after 3 h (Fig. 4A, middle panel). The delay of NXF induction time (with a peak at 3 h) observed in comparison with the ER stress case may be due to the fact that generation of oxidative radicals from SIN-1 in culture medium requires a few hours. Confirming the oxidative stress involvement in the NXF induction, the co-presence of SOD protein, a typical oxidative radical scavenger (25), abolished the induction of NXF by SIN-1 treatment, whereas control bovine serum albumin protein was without influence. Thirdly, osmotic stress induced by treatment with 1 M sorbitol (26) also activated NXF
expression (Fig. 4A, bottom panel). Thus several cell-degenerative stresses could induce NXF expression before they started to otherwise impair cells. Moreover, distinct up-regulation of NXF gene expression was found in an in vivo stroke model. A glutamate analogue, kainate, binds to glutamate receptors on neurons and induces specific brain damage derived from excessive excitation of neurons (glutamate excitotoxicity) (27). The same mechanism underlies neuron damage observed in stroke (28). As shown in Fig. 4B, in the kainate-treated mouse brain, significant NXF protein induction was detected in surviving neurons next to dying cells (Fig. 4B, top and middle panels). In contrast to NXF, a control protein, the phosphorylated form of Jun kinase (pJNK) (29), had exactly the reverse distribution and contrast to NXF, a control protein, the phosphorylated form of Jun kinase (pJNK) (29), had exactly the reverse distribution and

induction in surviving cells, not in dying cells, we performed anti-NXF immunostaining, TUNEL assay, and Fluoro-jade B staining on same brain slices sample (Brain challenged by transient global ischemia). The result shown in Fig. 4D indicates that there are few NXF protein-expressing cells in this damaged hippocampus (Anti-NXF). We can detect strong positive signals on this same brain slice with TUNEL assay and Fluoro-jade B staining instead.

The NXF-deficient Brain Shows Increased Susceptibility to a Typical Nerve Stress, Glutamate Excitotoxicity—Hypothesizing that the NXF-deficient mice have an impaired brain protective mechanism as the result of lacking on-demand inducible expression of NXF, we challenged NXF-deficient mice and control wild-type littermates with a typical glutamate receptor stimulator, kainate (27). In the 15-week-old wild-type mice, 28 mg/kg induced mild glutamate excitotoxicity accompanied by neuronal damage, particularly in the hippocampus, one of the most susceptible regions of the brain (20). A mild increase of GFAP immunoreactivity was also detected (Fig. 5, lower panel). The dose of kainate was not fatal for the wild-type mice. In contrast, three of eight 15-week-old NXF-deficient mice, with no obvious abnormality before the treatment, died within 5 days of the kainate injection, and the remainder exhibited severely abnormal behavior (myoclonic epilepsy) and serious brain damage with strong GFAP immunoreactivity (Fig. 5, upper panel). In particular, for example, K1, K2, and K3, which present relatively strong GFAP signals among other examples as shown Fig. 5, gave conspicuous Fluoro-jade B-positive signals in the hippocampus at CA1–2 region. Microglial cells, shows assembly of microglia in the hippocampus. Bottom panel: anti-nitrotyrosine antibodies react with oxidative species only in degenerated neurons in the NXF-deficient brain (cerebral cortex).
oxidative stress inducer, SIN-1, and an osmotic stress inducer, 1 M sorbitol, up-regulated NXF expression in vitro. We also observed induced expression of NXF in kainate (glutamate analogue)-treated brain and in degenerating brain with transient global ischemia. To determine whether the NXF expression is cell-protective or not, we challenged NXF-deficient mice (NXF/H11002/H11002) with an excitotoxicity inducer, kainate. The NXF-deficient brain showed elevated vulnerability to a typical nerve stress: glutamate excitotoxicity.

Taking our results together, we propose a hypothetical model for NXF functions in brain (Fig. 6). Neural stresses such as oxidative stress inducer, SIN-1, and an osmotic stress inducer, 1 M sorbitol, up-regulated NXF expression in vitro. We also observed induced expression of NXF in kainate (glutamate analogue)-treated brain and in degenerating brain with transient global ischemia. To determine whether the NXF expression is cell-protective or not, we challenged NXF-deficient mice (NXF−/−) with an excitotoxicity inducer, kainate. The NXF-deficient brain showed elevated vulnerability to a typical nerve stress: glutamate excitotoxicity.

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Neuronal cell stress
#Transient ischemia
#Glutamate stress...

↓

NXF mRNA induction

↓

Maintenance of oxidative stress-, ER stress-inducible response(s)
(Stress-inducible enzyme expression: GPX, XBP1, GRP78)

↓

Neural cell stress tolerance

↓

Neuronal damage; Apoptosis induction

FIGURE 6. A hypothetical model of NXF functions in brain. Neural stresses such as transient ischemia and glutamate excitotoxicity in daily life induce NXF mRNA. The induced NXF then renders host neurons tolerant to the neural cell stress.

as transient ischemia and glutamate excitotoxicity in daily life induce NXF mRNA, and the translated NXF protein contributes to the on demand cell protection under neural stresses. NXF-deficient mice (NXF<sup>−/−</sup>) lack this system and therefore exhibit increased susceptibility. Because NXF is a transcription factor, NXF-dependent cell protection should be executed by NXF target genes. Although XBP-1, GRP78, Ern1, and GPX2, which we found in this study to be candidates of indirect NXF target genes, could be important cell-protection players, direct NXF target genes need to be characterized in future studies.

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