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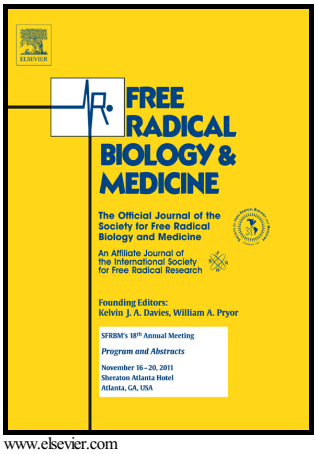


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# Circadian control of BDNF-mediated Nrf2 activation in astrocytes protects dopaminergic neurons from ferroptosis

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## Abstract

Astrocyte-neuron interactions protect neurons from iron-mediated toxicity. As dopamine can be metabolized to reactive quinones, dopaminergic neurons are susceptible to oxidative damage and ferroptosis-like induced cell death. Detoxification enzymes are required to protect neurons. Brain-derived neurotrophic factor (BDNF) plays a key role in the regulation of redox sensitive transcription factor Nrf2 in astrocytes and metabolic cooperation between astrocytes and neurons. This article reviews the importance of BDNF and astrocyte-neuron interactions in the protection of neurons against oxidative damages in rodent brains. We previously proposed that BDNF activates Nrf2 via the truncated TrkB.T1 and $p75^{NTR}$ receptor complex in astrocytes. Stimulation by BDNF generates the signaling molecule ceramide, which activates PKCζ leading to induction of the CK2-Nrf2 signaling axis. As a cell clock regulates $p75^{NTR}$ expression, we suggested that BDNF effectively activates Nrf2 in astrocytes during the rest phase. In contrast, neurons express both TrkB.FL and TrkB.T1, and TrkB.FL tyrosine kinase

activity inhibits $p75^{NTR}$-dependent ceramide generation and internalizes $p75^{NTR}$. Therefore, BDNF may not effectively activate Nrf2 in neurons. Notably, neurons only weakly activate detoxification and antioxidant enzymes/proteins via the Nrf2-ARE signaling axis. Thus, astrocytes may provide relevant transcripts and/or proteins to neurons via microparticles/exosomes increasing neuronal resistance to oxidative stress. Circadian increases in the levels of circulating glucocorticoids may further facilitate material transfer from astrocytes to neurons via the stimulation of pannexin 1 channels-P2X7R signaling pathway in astrocytes at the beginning of the active phase. Dysregulation of astrocyte-neuron interactions could therefore contribute to the pathogenesis of neurodegenerative diseases including Parkinson's disease.

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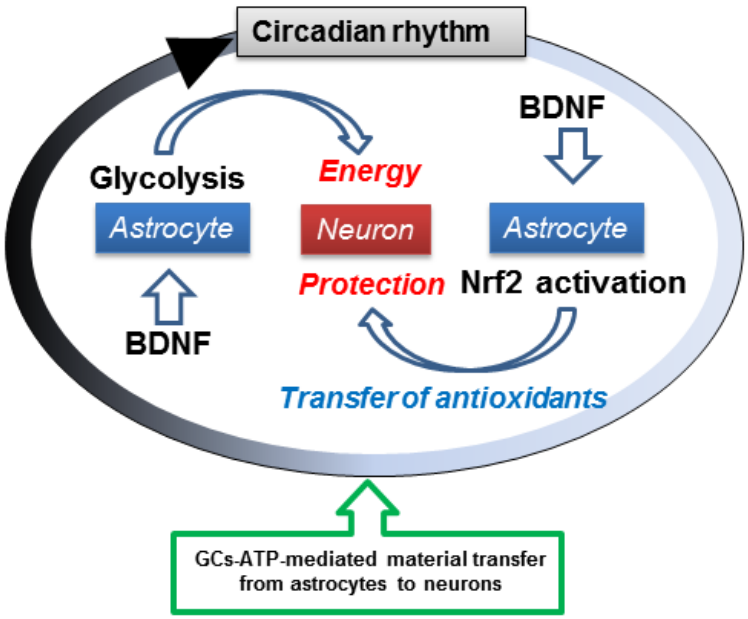


Graphical abstract

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Key words:

ferroptosis, dopamine, Nrf2, BDNF, circadian rhythm, TrkB, $p75^{NTR}$, cystine transport, GSH, mitochondria, CK2, PKA, Parkinson's disease

**List of Abbreviations**

ATF4, activating transcription factor 4; BDNF, brain-derived neurotrophic factor; CK2, casein kinase 2; Cry, cryptochrome; FGF2, fibroblast growth factor-2; GST, glutathione-*S*-transferase; NGF, nerve growth factor; Nrf2, nuclear factor-E2-related factor 2; NQO1, NAD(P)H quinone oxidoreductase; NMDAR, M-methyl-*D*-aspartate-receptor; PKA, protein kinase A; $p75^{NTR}$, p75 neurotrophin receptor; Per2, period 2; PP1, protein phosphatase-1; SCN, suprachiasmatic nucleus; tBHQ, *tert*-butyl-hydroquinone; tPA, tissue-type plasminogen activator; Trk, tropomyosin-related kinase; ZT, Zeitgeber time

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## 1. Introduction

Ferroptosis is defined as a form of cell death characterized by iron-dependent accumulation of lipid hydroperoxides to lethal levels. Aberrations in brain iron homeostasis have been implicated in pathological cell death associated with various diseases including neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (reviewed in [1-3]). Iron plays a fundamental role in the development of the central nervous system as well as in several neuronal functions including synaptic plasticity [4]. Astrocytes have been demonstrated to serve critical functions in the protection of neurons from iron overload. Non-transferrin-bound iron is taken up via resident transient receptor potential channels in quiescent astrocytes and the *de novo*

expressed divalent metal transporter 1 in activated astrocytes [5-7]. Astrocytes through these routes accumulate iron and various iron-containing compounds, store iron efficiently in ferritin and also export iron (reviwed in [4,8,9]). Notably, serum iron levels and expression of divalent metal transporter 1, transferrin receptor and other iron related gene expression in tissues exhibit diurnal variation [10].

Parkinson's disease is caused by neuronal loss in multiple brain regions, especially dopaminergic neurons in the substantia nigra pars compacta. Although it is difficult to pinpoint the type of neuronal death involved [11], biochemically, Parkinson's disease is characterized by mitochondrial dysfunction, accumulation of iron, diminished copper content and depleted glutathione (GSH) levels in these regions [12,13]. These symptoms suggest the existence of iron dyshomeostasis and iron-mediated oxidative damage. Preventing neuronal death is a major strategy for disease-modifying therapies, but it is currently unknown what causes iron overload and the defects in detoxification systems in the disease (reviewed in [14,15]).

As dopamine metabolism produces reactive *o*-quinones, dopaminergic neurons are especially susceptible to oxidative stress and mitochondrial dysfunction [16]. Tyrosine hydroxylase is the rate-limiting enzyme in dopamine synthesis from tyrosine. In neurons, a part of dopamine is oxidized to dopamine *o*-quinone and then metabolized to generate aminochrome, which causes neurotoxicity [16]. Notably, some quinones including 9,10-phenanthraquinone are highly toxic inducing ferroptosis-like cell death [17]. Therefore, removal of aminochrome is essential for the protection of neurons. Glutathione *S*-transferase mu 2 (GSTM2) plays a key role in detoxifying *o*-quinones [16]. Aminochrome is further metabolized by NAD(P)H quinone oxidoreductase (NQO1/DT-diaphorase) leading to the formation of neuromelanin, which chelates iron

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and other metals [18] protecting neurons from oxidative stress (Fig. 1 A). As the contents of ferritins are low in neurons, neuromelanin is the major iron storage in substantia nigra neurons in normal individuals [19]. Notably, iron is required for the dynamics of striatal dopamine, as low substantia nigra iron is a known pathology of Restless Legs Syndrome, which accompanies increases in extracellular striatal dopamine due to a defect in a dopamine receptor 2 mediated feedback inhibition of dopamine release [20].

Synthesis of the iron-containing prosthetic group-heme and iron-sulfur clusters occurs in mitochondria. Mitochondria generate reactive oxygen species as byproducts of molecular oxygen consumption in the electron transport chain. Superoxide anion and hydrogen peroxide produced in mitochondria are precursors of hydroxyl radical through the participation of transition metals [21,22]. Glutathione (GSH) and GSH-dependent detoxification enzymes play a major role in the elimination of the hydroperoxides in mitochondria as well as in the cytoplasm. GSH is synthesized in the cytoplasm and sequestered in mitochondria by the action of a membrane carrier that transports GSH into mitochondrial matrix [23]. The intracellular cysteine pool limits the synthesis of GSH. Astrocytes induce cystine transport activity to maintain intracellular GSH levels in culture [24]. In contrast, neurons do not express cystine transport activity and depend on cysteine transport for the synthesis and maintenance of intracellular GSH levels [24, 25].

To adapt to oxidative stress conditions, cells activate the transcription factor nuclear factor-E2-related factor 2 (Nrf2) which regulates the expression of a large number of genes, including detoxification enzymes, antioxidant proteins and GSH related genes via the antioxidant responsive element (ARE) in a cell type specific manner [26-28]. As

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the brain consumes glucose and oxygen at high rates, dysregulation of the Nrf2-ARE defense system is associated with oxidative damage and ultimately neurodegeneration. Activation of Nrf2 in mouse brain astrocytes protect cells from cytotoxicity induced by iron overload [29]. Recent studies have identified that several genes involved in heme synthesis, hemoglobin catabolism, iron storage, and iron export are under the control of Nrf2 [30].

Brain-derived neurotrophic factor (BDNF) is the major neurotrophin expressed in adult brain and has been implicated in regulating neuronal survival, differentiation, and synaptic plasticity. We recently proposed that BDNF plays a key role in circadian activation of Nrf2 in astrocytes and suggested that BDNF activates Nrf2 via receptor combination of the truncated form of TrkB.T1 and the low affinity receptor $p75^{NTR}$, which generate the lipid signal mediator ceramide upon stimulation [31]. As $p75^{NTR}$ expression is directly controlled by the cell clock core transcription factor complex Clock-Bmal1, we thought activation of Nrf2 via BDNF depends on circadian rhythm [28]. BDNF plays a key role in the maintenance of dopaminergic neurons in the substantia nigra pars compacta in mice [32], but how BDNF protects neurons is not fully elucidated.

In this review, we discuss the importance of circadian rhythm in BDNF-mediated metabolic cooperation between astrocytes and neurons, and the role of Nrf2 activation in astrocytes in the protection of neurons against ferroptosis-like cell death (Fig. 1 B). As neurons express both full-length and truncated forms of TrkB, TrkB.FL and TrkB.T1, the signaling pathways regulated by BDNF are much more complicated compared to those in astrocytes. Another neuron-specific feature is that the Nrf2-ARE signaling axis upregulates a totally different set of genes compared to astrocytes. Although activation

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of the Nrf2-ARE axis upregulates classical detoxification and antioxidant enzymes/proteins in astrocytes, neurons hardly express these genes upon Nrf2 activation, rendering neurons highly susceptible to oxidative damages (Table 1) [33]. These differences between neurons and astrocytes raise questions as to whether BDNF plays a role in Nrf2 activation in neurons and how neurons are protected against oxidative stress without the ability to upregulate expression of key cellular antioxidants?

## 2. GSH-dependent protection of brain cells

Ferroptosis can easily be induced in cultured cells by depleting cellular GSH. Bannai et al. [34] first observed this phenomenon in human diploid fibroblasts cultured in a medium deficient in cystine. Elegant studies by Bannai and colleagues revealed that cellular GSH readily effluxes from fibroblasts, with synthesis of GSH from precursor amino acids required to maintain intracellular GSH levels and the cysteine pool limiting GSH synthesis. These researchers identified a membrane transport system highly specific for cystine and glutamate [35], which plays a key role in the maintenance of cellular cysteine and GSH in cultured fibroblasts (reviewed in [25,36]). Cystine transport activity was termed system $x_c^-$ and is induced in many cell types including fibroblasts, macrophages and astrocytes during culture *in vitro*. However, other cells such as neurons and lymphocytes can hardly induce cystine transport activity in response to oxidative stress. The latter cells depend on cysteine uptake for maintenance of cellular GSH and can only survive in standard culture media when co-cultured with system $x_c^-$ expressing cells, known to secrete and provide cysteine to recipient cells [24,25].

The expression of the cystine transporter (xCT), a component of cystine-glutamate

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exchange system $x_c$-, is observed in some specific regions of the adult brain [37,38]. Notably, a subpopulation of astrocytes are labeled with an xCT antibody, but with greatly varying intensities [39]. However, deficiency of xCT in mice is associated with no apparent problems under standard breeding conditions [37,38]. Notably, plasma levels of cystine are higher in xCT deficient mice compared to normal mice, but other amino acids including cysteine, glycine and glutamate are similar between xCT deficient and wild type mice. These results suggest that deficiency in cystine transport activity itself does not cause GSH depletion in cells and tissues in mice under normal conditions [38].

Nrf2 positively regulates the activity of system $x_c$- or gene expression of xCT in cultured macrophages and other cells types [25,40]. Activating transcription factor 4 (ATF4), which is increased in response to a diverse array of microenvironmental stresses, including amino acid depletion and endoplasmic reticulum stress, also regulates xCT gene expression [41]. Notably, an increase in the cystine/glutamate exchanger in astrocytes does not always protect co-cultured neurons, as enhanced glutamate efflux may cause excitotoxicity in neurons. Liu et al. [42] observed that fibroblast growth factor-2 (FGF-2) upregulates system $x_c$- activity in astrocytes with increased glutamate release causing AMPA/kainate receptor mediated toxicity. As FGF-2 can activate ATF4 [43,44], the effect of FGF-2 on upregulation of system $x_c$- activity may partly depend on ATF4. Notably, the protein level of ATF4 is upregulated in both Alzheimer's disease patients and the disease model in mouse brain [45]. In contrast to ATF4, Nrf2 protects neurons from kainite toxicity, highlighting the importance of the Nrf2-ARE dependent detoxification system in addition to GSH for the protection of neurons from glutamate excitotoxicity [46].

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GSH transferases (GSTs) play important roles in eliminating toxic metabolites. GSTM2 has much higher activity than other GSTs in catalyzing the GSH conjugation of aminochrome and its precursor dopamine *o*-quinone [47] (Fig. 1 A). GSTM2 is expressed in astrocytes but not synthesized in dopaminergic neurons. As astrocytes have been shown to provide GSTM2 to neurons to help protect against neurotoxicity of aminochrome, this implicates an intercellular communication system mediating transfer of cytoplasmic proteins from astrocytes to neurons [16,48]. In addition to GSTs, GSH peroxidases (GPxs) reduce phospholipid hydroperoxides and afford protection for neurons. GSH peroxidase 4 (GPx4) is a selenoenzyme and long and short isoforms of GPx4 are generated from the GPx4 gene. Ferroptosis-associated markers such as elevated lipid peroxidation, ERK activation and augmented neuroinflammation, have been observed in GPx4 deficient mice brain [49]. Peroxiredoxin 6 is a bifunctional enzyme with both GSH peroxidase and acidic $Ca^{2+}$-independent phospholipase $A_2$ activities [50]. When mitochondrial membranes are damaged by oxidative stress, Prx6 translocates to depolarized mitochondria from the cytoplasm to limit mitochondrial dysfunction and regulate the initial stage of mitophagy [51,52].

## 3. Nrf2 activates different genes in astrocytes and neurons

Nrf2 plays a key role in the protection of cells and tissues from oxidative damage via upregulation of detoxification enzymes, including GSTs, NQO1, antioxidant enzymes such as heme oxygenase-1, peroxiredoxin 1, and cystine transport activity [26,27]. Nrf2 also regulates expression of iron storage protein ferritin [53]. Recent studies demonstrated that administration of Nrf2 activators that cross the blood-brain barrier, such as dimethyl fumarate and fumaric acid esters, protect neurons against oxidative

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damage caused by neurotoxins such as 6-hydroxidopamine and 1-metyl-4-phenyl-1,2,3,6-tetrahydropyridine, used in animal models of Parkinson's disease [54,55].

However, it is important to highlight that Nrf2 target genes in neurons are quite different from those expressed in astrocytes. Kraft et al. [33] analyzed Nrf2-dependent gene expression separately in neurons and astrocytes freshly prepared from mouse embryonic brains. These authors treated primary cultured cells with the well-known Nrf2 activator *tert*-butyl-hydroquinone (tBHQ) and found that astrocytes upregulate typical Nrf2-dependent genes including detoxification enzymes including GSTs, NQO1, ferritin and GSH synthetase in addition to glucose metabolism-related genes (Table 1). In contrast, most of these genes are hardly upregulated in neurons by tBHQ [33]. Of the 97 genes increased in the astrocyte-enriched population, only 4 transcripts (glutathione-*S*-transferase mu1, glucose phosphate isomerase 1, malate NADP oxidoreductase and neuroleukin) are also increased in neurons. In neurons, some of the upregulated genes are related to mitochondrial activation, calcium binding and transport, cell adhesion and integrin signaling and synaptic activity (Table 1). One of the Nrf2 upregulated genes, mitochondrial creatine kinase, provides a temporal and spatial energy buffer to maintain cellular energy homeostasis. Ndufa1 encodes one of the proteins composing mitochondrial complex I (the proton-translocating NADH-quinone oxidoreductase), exhibiting an essential role for the complex I activity [56]. S100A1 is a $Ca^{2+}$-sensing protein of the EF-hand family and interacts with mitochondrial $F_1$-ATPase and enhances ATP synthesis [57]. Thus, activation of Nrf2 apparently increases mitochondrial activity in neurons, and yet fails to induce classical Nrf2-regulated antioxidants and detoxification enzymes. These results raise the question as to how

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neurons manage to cope with oxidative stress following activation of mitochondria?

## 4. Neurotrophins can activate Nrf2-ARE signaling axis

Neurotrophins such as NGF and BDNF play important roles in the survival and function of neurons and astrocytes. Previously, Kosaka et al. [58] showed that NGF activates Nrf2 in rat pheochromocytomatous PC12 cells, however, the precise mechanism of NGF-mediated Nrf2 activation remains to be elucidated. Dobrowsky et al. [59] were the first to report that stimulation of the low affinity neurotrophin receptor $p75^{NTR}$ activates neutral sphingomyelinase to generate ceramide. Blöchl and Sirrenberg [60] measured ceramide generation in rat mesencephalic neurons in culture following stimulation either with NGF or BDNF, with NGF generating ceramide levels about 3-fold higher than control. As neurons express very low levels of TrkA, these authors concluded that NGF generated ceramide occurs through direct interaction with $p75^{NTR}$. Although high levels of ceramide are toxic, physiologically low levels of ceramide can activate atypical protein kinase C ζ (PKCζ) [61]. As PKCζ can activate CK2, we proposed that neurotrophins are able to activate $p75^{NTR}$-ceramide-PKCζ-CK2 signaling pathway. Subsequently, CK2 directly phosphorylates and stabilizes/activates Nrf2 [31,62,63] (Fig. 2 A).

In contrast to NGF, BDNF only marginally increases ceramide under similar culture conditions irrespective of the fact that the neurons express full length TrkB (TrkB.FL). However, pretreatment of neurons with the tyrosine kinase inhibitor K252a increases ceramide generation, suggesting that tyrosine kinase activity of TrkB.FL inhibits $p75^{NTR}$-mediated activation of neutral sphingomyelinase [60]. Their study indicates that the BDNF-TrkB.FL-$p75^{NTR}$ axis does not induce ceramide-dependent Nrf2 activation in

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neurons. Their study further suggested that the truncated form of TrkB (TrkB.T1), lacking the intracellular receptor tyrosine kinase domain, does not inhibit ceramide generation via $p75^{NTR}$ (Fig. 2 A). As astrocytes predominantly express TrkB.T1, we proposed that the BDNF-TrkB.T1-$p75^{NTR}$ mediated generation of ceramide triggers the PKCζ-CK2-Nrf2 signaling pathway [31].

However, $p75^{NTR}$ is a clock component and expression is under the direct control of the Clock-Bmal1 core transcription factor complex [64]. The Clock-Bmal1 complex regulates expression of negative transcriptional regulators, including Cry, Period and Rev-erb thereby forming a negative feedback loop. Cry and Period proteins form a regulatory complex which inhibits Clock-Bmal1 transactivation of target genes and the nuclear receptors Rev-erb α and β are transcriptional repressors and repress BMAL1 transcription [65,66] (Fig. 2 B). Therefore, the $p75^{NTR}$-mediated signaling pathway should change depending on the circadian rhythm in astrocytes. As period 2 (Per2) levels are high in the early light/rest phase in local regions of rat brain [67,68] (Fig. 2 B), we predicted that the $p75^{NTR}$-Nrf2 signaling pathway also effectively functions in the similar time phase [31]. Currently, however, the circadian variation of $p75^{NTR}$ protein in brain cells has yet to be investigated.

Dopamine release in the brain has a daily rhythm peaking during the dark/active phase in rodents, which is significantly different from that of Per2 [67] (Fig. 2 B). Although the underlying mechanisms for regulation of extracellular dopamine rhythm are not known, repression of tyrosine dehydrogenase gene transcription by Rev-erbα may partly modulate the dopamine rhythm [69,70]. Thus, dopaminergic neurons should be fully protected from toxicity of the dopamine metabolites during the active/dark phase.

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## 5. Differences in BDNF signaling in astrocytes and neurons

Circadian rhythm differentially regulates metabolic activity in astrocytes and neurons to share oxygen consumption and to effectively utilize glucose and its metabolites. We proposed that the BDNF-TrkB.T1-$p75^{NTR}$-ceramide signaling axis activates the PKCζ-CK2-Nrf2 pathway mainly in the light/rest phase in rodent astrocytes [31]. Notably, CK2 also facilitates protein import into the mitochondrial matrix to enhance oxidative phosphorylation [71]. Ceramide also activates protein phosphatase 1 (PP1) [72], which plays a key role in glycogen synthesis [73]. Therefore, when neuronal activity remains low, astrocytes favorably synthesize glycogen to store energy which consumes ATP and requires mitochondrial activity. But in the dark/active phase, when neuronal activity increases, astrocytes undergo a metabolic shift towards increasing glycogen hydrolysis and glycolysis to produce lactate as a substrate for the TCA cycle in neurons. This phase change can occur simultaneously with the down-regulation of $p75^{NTR}$ which results in the change of the functional partner of TrkB.T1 from $p75^{NTR}$ to adenosine receptor $A_{2A}$ ($A_{2A}R$) in astrocytes [31]. Thereafter $A_{2A}R$-mediated cAMP/PKA signaling dominates over ceramide-mediated CK2 and Nrf2 activities in the active phase. In contrast to CK2, PKA suppresses protein import into mitochondria leading to downregulation of oxidative phosphorylation [71] (Fig. 3 A).

In contrast to astrocytes, neurons express both TrkB.FL and TrkB.T1 receptors, and BDNF-TrkB.FL-$p75^{NTR}$ signaling does not support ceramide-CK2 dependent Nrf2 activation. Additionally, BDNF-TrkB.FL signaling induces internalization and accumulation of $p75^{NTR}$ in exosome-related multi-vesicular bodies MVBs without rapid degradation in neurons, and $p75^{NTR}$-loaded exosomes can be released when neuronal

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activity increases [74]. The expression levels of both types of TrkB.FL and TrkB.T1 vary among neurons, and neuronal damage induces changes in the ratio of the two receptors [75]. Therefore, it is not clear at present whether the BDNF-TrkB.T1-$p75^{NTR}$ axis contributes to activation of transcription factor Nrf2 in neurons *in vivo* [76]. However, if activation of BDNF-TrkB.T1-$p75^{NTR}$ signaling occurs locally along axons distal to soma, it may contribute elongation of distal dendrites [77], and ceramide-activated PP1 may play a role in the regulation of neurofilaments and transport of cargoes along axons in neurons [78,79] (Fig. 3 B).

## 6. Neurons activate Nrf2 in astrocytes in a mixed culture

Habas et al. [80] showed that an interaction of astrocytes and neurons is important in the regulation of Nrf2 activation in astrocytes. The authors cultured neurons and astrocytes prepared from rat embryo brain hippocampus and observed that stimulation of neurons with $GABA_A$ receptor antagonist gabazine and/or $K^+$ channel inhibitor 4-aminopyridine (4-AP), which induces depolarization and neurotransmitter release, induced Nrf2 activation in astrocytes but not in neurons. As the same treatment did not cause Nrf2 activation in astrocyte predominant culture, the authors suggested soluble factors released from stimulated neurons induced Nrf2 activation in astrocytes. It was also shown that release of glutamate and activation of group I metabotropic glutamate receptors (mGluR1 or mGluR5) during the neuronal stimulation plays a role in the astrocyte-specific activation of Nrf2.

Although Habas et al. [80] did not identify the factors that cause Nrf2 activation in astrocytes, we propose BDNF as the major Nrf2 activation factor released during co-culture following depolarizing stimulation of neurons. BDNF is the main

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neurotrophin in the brain and can be released from neurons in an activity-dependent manner [81]. In the BDNF-rich hippocampal circuit, BDNF is highly abundant in presynaptic terminals of glutamatergic neurons. Activation of presynaptic and postsynaptic glutamate receptors AMPAR and NMDAR is important for BDNF release from nerve terminals [82,83]. Notably, BDNF is synthesized as a precursor (proBDNF) and is secreted as either a mature furin-processed form or an unprocessed pro-form. In the hippocampus, secreted proBDNF is cleaved to generate mature BDNF by the extracellular serine protease, plasmin, which is produced from plasminogen by tissue-type plasminogen activator (tPA) [84,85]. Notably, activation of metabotropic glutamate receptor upregulates tPA protein synthesis [86]. However, cultured astrocytes efficiently secrete plasminogen activator inhibitor-1 (PAI-1) and suppress generation of plasmin [87]. Taken together, multiple glutamate receptors are involved in the control of proBDNF secretion from stimulated neurons and additional factors are involved in the generation of active mature BDNF. Additionally, astrocytes can absorb proBDNF released from neurons via its receptor $p75^{NTR}$ to store proBDNF in intracellular vesicles and proBDNF is processed to mature BDNF before endocytic BDNF secretion [84,88]. However, depolarization does not secrete BDNF from astrocytes. The presence of these multiple steps may be the reason why it takes overnight incubation to accumulate mature BDNF in the medium to induce Nrf2 activation via the TrkB.T1-$p75^{NTR}$ axis in astrocytes following stimulation of neurons (Fig. 4 A).

Notably, Habas et al. [80] also showed that Nrf2 activation in astrocytes can be observed as early as in 2 h when the mixed culture was stimulated with 50 mmole/L $K^+$. This effect of high $K^+$ is caused by the different mechanism from the depolarization of neurons. We propose this effect was caused by mature BDNF released from astrocytes

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following treatment with high $K^+$. It was shown by others that stimulation with 40 mmole/L $K^+$ induces secretion of stored mature BDNF from astrocytes within 5 min [88]. Released mature BDNF can directly activate Nrf2 in astrocytes via BDNF-TRkB.T1-$p75^{NTR}$ axis. Although, high $K^+$ is an artificial condition, ATP can also induce rapid release of BDNF from astrocytes [88]. As astrocytes are known to secrete ATP through hemichannels, ATP-mediated rapid BDNF release from astrocytes seems to be a physiological response (Fig. 4 A). Pannexin 1 channels can be activated by elevations in extracellular $K^+$ [89]. High $K^+$ induces a shift of activation potentials to more physiological range, thereby allowing channel opening at resting or slightly depolarized potentials [90] (Table 2). Therefore, it is plausible that high $K^+$ opens pannexin 1 channels releasing ATP, leading to release of BDNF by the mechanism discussed in the next section. In conclusion, our hypothesis that BDNF synthesized in hippocampal neurons can activate Nrf2 in astrocytes extends the observations reported by Habas et al. [80].

## 7. ATP induces release of BDNF and microparticles from astrocytes

ATP is a widespread cell-to-cell signaling molecule in the brain and pannexin 1 hemichannels are one of the major pathways mediating ATP release from astrocytes (reviewed in [91,92]. In the plasma membrane, Pannexin 1 forms a hexamer composing a transmembrane channel which mediates ATP release under physiological and pathological conditions. Each Pannexin 1 has a low affinity ATP binding site, which functions as a negative feedback mechanism for ATP release to prevent cell toxicity. Increased $Ca^{2+}$ influx induces opening of the pannexin 1 channels leading to release of ATP from astrocytes (Table 2). As pannexin 1 channels have a large pore size and an

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apparent lack of selectivity, other small molecules of the size of ATP such as glutamate and D-serine will pass through the channels, but we focus here on ATP.

ATP released from astrocytes plays a key role for the release of BDNF [88] and microparticles [93]. Astrocytes are known to actively release divergent secretory organelles, including synaptic-like microvesicles and exosomes to communicate with other cells including neurons (reviewed in [94-96]). Exosomes are small vesicles generated by the trafficking of MVBs from the cytosol to the cell surface carrying microRNAs, mRNAs, proteins and lipids. The second type of extracellular vesicles, termed microvesicles or ectosomes are derived from the plasma membrane and are released in response to specific stimuli, such as changes in extracellular ATP levels [97,98].

P2X family purinergic receptors are membrane ion channels preferably permeable to sodium, potassium and calcium that open within milliseconds of ATP binding. Therefore, over-activation of P2X channels with unphysiological, high concentrations of ATP induces cell death. Ectonucleotidases hydrolyze released ATP to adenosine protecting neuronal cells from over-activation of P2X receptors. Microglial cells and astrocytes in the substantia nigra in rats express P2X7 receptors. However, dopaminergic neurons in the substantia nigra compact in adult rat express P2X1-6R, but not P2X7R [91]. Activation of the P2X7R with low levels of ATP triggers a remarkably diverse array of membrane trafficking responses (reviewed in [99,100]). These responses can result in the release of bioactive proteins, including mature BDNF, lipids, and large membrane complexes into extracellular compartments for communication with other cells. P2X7R regulates shedding of plasma membrane surface proteins, exocytosis of secretory lysosomes and release of exosomes from multivesicular bodies.

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## 8. Transfer of membrane vesicles to neurons

As astrocytes are known to actively release divergent secretory organelles, including synaptic-like microvesicles and exosomes [101,102], it is plausible that astrocytes systematically and time-dependently provide various molecules, including Nrf2-regulated gene products and/or transcripts, to neurons to protect them against oxidative stress. Notably, GSTM2, a key enzyme for detoxification of dopamine *o*-quinones, can be transferred from glioblastoma U373MG cells to neuronal SH-SY5Y cells in culture to protect the neuronal cells from reactive quinones [48].

Proteomics analysis has revealed that exosomes released from different types of cultured cells contain various proteins. Notably, among the top 50 most commonly identified proteins in exosome preparations [94], 5 target genes of the Nrf2-ARE axis actually found among upregulated genes in astrocytes stimulated with tBHQ [33]. These are glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, phosphoglycerate kinase 1, triosephosphate isomerase and peroxiredoxin-1, with two of them highlighted in Table 1. Peroxiredoxins 1 and 2, and thioredoxin 1 are constantly released from various cultured cells under mild oxidative stress [103]. These studies suggest that secretion of GSTM2 from astrocytes probably depends on exosomes [48]. Ferritin is also secreted via the multivesicular body-exosome pathway [104]. Astrocyte-specific exosomes contain sortilin known as the co-receptor for $p75^{NTR}$ in the proNGF- and proBDNF-induced cell death, and moreover glioblastoma cells release TrkB-containing exosomes [105]. Microvesicles from astrocytes also contain different types of proteins including Cu-Zn SOD and matrix metallopeptidases [106]. Taken together astrocytes can release some of the Nrf2-regulated gene products, GSTM2 and also BDNF

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receptors $p75^{NTR}$ and/or TrkB.T1 via exosomes and microvesicles. Further studies are warranted to identify the contents in these membrane particles and whether these modulate neuronal function.

Neurons can physically interact with astrocytes through membrane proteins and mutually stimulate intracellular signaling. Thy-1 is a membrane glycoprotein expressed in neurons, T cells, endothelial cells and some other cell types. Thy-1 interacts with αvβ3 integrin and syndecan-4 expressed in astrocytes [107,108]. Thy-1 binding to αvβ3 integrin on astrocytes triggers calcium transient and activates purinergic receptor P2X7R-mediated calcium entry in astrocytes [109] (Table 2). However, primary astrocytes express low levels of αvβ3 integrin and are unresponsive to Thy-1, suggesting additional factors are involved in the regulation of intercellular communication [110]. Notably, specific interaction of these two membrane proteins is used for targeted delivery of αvβ3 integrin containing extracellular vesicles to Thy-1 expressing recipient cells [111-113]. These studies suggest that neurons may have the potential to efficiently receive extracellular membrane vesicles containing αvβ3 integrin released from nearby astrocytes using Thy-1 as the receptor.

As neurons upregulate expression of quite different set of Nrf2 target genes compared to those in astrocytes (Table 1), it is a plausible scenario that astrocytes transfer to neurons a set of enzymes and proteins to support metabolic shifts and to protect them from oxidative damage during the active/dark phase. The transfer of these enzymes and proteins could instantly afford protection for neurons against oxidative stress. If our hypothesis is correct, the process is time- and energy-efficient compared to synthesis of proteins in the soma and transport along axons to appropriate targets. This process would also enable astrocytes to release unnecessary cell components via membrane

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vesicles resulting in the shift of energy metabolism.

## 9. Glucocorticoids regulate circadian control of ATP release from astrocytes

Release of ATP from astrocytes under physiological conditions is regulated by circadian rhythm [114-116]. Primary cultures of astrocytes express circadian oscillations in extracellular ATP accumulation that persist for multiple cycles with periods of about 23 h [114,115]. ATP levels in the rat SCN *in vivo* are also marked by rhythmic variation during exposure to 12 h of light and 12 h of dark, with peak accumulation occurring during the latter half of the dark phase or subjective night. Notably, the master clock in SCN is controlled by light signals and entrains subsidiary oscillators in other brain regions as well as many peripheral tissues. These peripheral clocks have significantly different time phases compared to the master clock in nocturnal animals such as rats and mice [117,118].

Notably, release of ATP in the brain is controlled by plasma levels of glucocorticoids (GCs). The time of the day-dependent changes in the secretion of GCs from the adrenal glands are controlled by the SCN through the Hypothalamus-Pituitary-Adrenal neuroendocrine axis [119] (Fig. 5 A). GCs released from the adrenal glands play a key role in synchronizing subsidiary oscillators to coordinate various biological processes, thereby entraining daily rhythms in physiology and behavior [120,121]. The glucocorticoid receptor (GCR) is expressed in most cell types and GCs-GCR signaling induces various effects in a cell-type dependent manner [122]. Recently, Koyanagi et al. [123] showed GCs stimulates ATP release from spinal astrocytes in mice. Plasma levels of GC/corticosterone have a sharp peak before beginning of the dark/active phase and the extracellular ATP in the cerebrospinal fluid markedly increased in almost the same

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time phase as corticosterone (Fig. 5 A). These authors showed that GC-GCR axis opens pannexin 1 channels in astrocytes, largely mediated by the activation and upregulation of serum- and glucocorticoid-enhanced kinase-1 (SGK-1). They further discussed that SGK-1 increases store-operated calcium entry via activation of Orai-1, a plasma membrane channel forming unit [123].

Thus GCs-mediated sharp increases in ATP via opening of pannexin 1 channels in astrocytes in the brain occur just before the phase change from light/rest to dark/active phase in mice [123]. Increased extracellular ATP will facilitate release of BDNF and microparticles and exosomes from astrocytes (Fig. 4 B). As a result, BDNF stimulates neurons and astrocytes via autocrine/paracrine loop and microparticles and exosomes are transferred from astrocytes to neurons (Fig. 5 B). Thus, we speculate daily increases in plasma GCs stimulate astrocyte-neuron interactions in the brain to facilitate and to harmonize circadian phase changes, vitalizing neurons and increasing the 'nursing activity' of astrocytes through systematic material transfer from astrocytes to neurons (Fig. 4 B and 5).

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**10. Summary and future perspectives**

Astrocyte-neuron interactions play a key role in iron metabolism and protection of neurons from ferroptosis. Astrocytes have a high capacity to store iron and prevent iron overload in neurons (reviewed in [4,8,9]), in part dependent on the activation of the Nrf2 defense pathway [29,30]. Astrocytes provide neurons with GSTM2 and other antioxidants to afford protection from oxidative damage. Dysregulation of astrocyte-neuron interactions and inadequate Nrf2 activation in astrocytes may lead to ferroptosis-like cell death in neurons especially dopaminergic neurons. We propose

BDNF plays a key role in coordinating astrocyte-neuron interactions and the circadian regulation of Nrf2 expression and mitochondrial activity in astrocytes. In contrast, BDNF may not effectively activate Nrf2 in neurons, therefore the mechanism and importance of Nrf2 activation in neurons *in vivo* remains unresolved.

In this review, we discussed the importance of pannexin 1 hemichannels in the regulation of intercellular communication between astrocytes and neurons. Pannexin 1 channels are expressed wide variety of cells and released ATP transduce signals through its receptors. The physical interaction of astrocytes and neurons via αvβ3 integrin and Thy-1 opens pannexin 1 channels [109]. In addition to direct interaction of astrocytes with neurons, adrenal stress hormone GCs play a key role in the circadian control of pannexin 1 channels and release of ATP from astrocytes in the brain [123]. Thus, GCs coordinate the metabolic shift from the rest into the active phase in rodents.

Circadian regulation of $p75^{NTR}$ must play a key role in time-dependent alteration of BDNF-mediated signaling pathways and metabolic cooperation between astrocytes and neurons. Notably, over- or chronic-activation of BDNF-TrkB.T1-$p75^{NTR}$ signaling in astrocytes does not favor generation of lactate to support neurons (Fig. 3 A). In contrast, insufficient $p75^{NTR}$-CK2-Nrf2 signaling in astrocytes may not protect neurons from oxidative damage.

Disturbances in circadian rhythms and sleep-awake cycles have been considered as symptoms of aging-related neurodegenerative conditions such as Alzheimer's, Parkinson's and Huntington disease (reviewed in [124-127]). Clinical studies and experiments in animal models of neurodegenerative disorders have revealed the progressive nature of circadian dysfunction throughout the course of these diseases. Aging has been associated with iron retention in many cell types, and in neurons,

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promoting neurodegeneration by ferroptosis [128], which may partly be due to circadian dysregulation. As disruption of the circadian rhythm may be associated with inadequate activation of Nrf2 and dysregulation of astrocyte-neuron interactions, control of the circadian clock and sleep-wake cycles are particularly important for the normalization of brain functions and neuronal protection.

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**Fig. 1. Protection of dopaminergic neurons from oxidative stress.** ***A***, Tyrosine



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dehydrogenase is the key enzyme for dopamine synthesis from tyrosine. Dopaminergic neurons are highly susceptible to oxidative stress as dopamine can be metabolized to produce reactive quinones. Both GSTM2 and NQO1/DT-diaphorase are required to effectively detoxify quinones to protect the neurons [16]. The dopamine metabolite neuromelanin protects neurons. ***B***, BDNF regulates metabolism in neurons and coordinates metabolic interactions between neurons and astrocytes. Astrocytes interact with neurons and support their survival and activity in various ways. Circadian rhythm controls daily changes of BDNF-TrkB signaling pathways in astrocytes thereby managing phase-dependent interaction between astrocytes and neurons.

**Fig. 2. NGF- and BDNF-mediated activation of Nrf2 and circadian rhythm.** ***A***, Stimulation of BDNF through full length receptor TrkB.FL with the tyrosine kinase domain suppresses $p75^{NTR}$-mediated ceramide generation and does not activate Nrf2. Truncated receptor TrkB.T1 without tyrosine kinase domain can activate Nrf2 via the BDNF-TrkB.T1-$p75^{NTR}$-ceramide-CK2 signaling pathway [31,76]. NGF has a higher affinity than BDNF for $p75^{NTR}$ and can stimulate $p75^{NTR}$-ceramide-CK2-Nrf2 signaling pathway. As Nrf2 can upregulate NGF gene expression, a positive feedback loop between Nrf2 and NGF can be formed. ***B***, The transcription factor complex Clock-Bmal1 regulates expression of Per2, $p75^{NTR}$, Rev-erbα and others. Per2 suppresses the activity of the Clock-Bmal1 forming a negative feedback loop to make a daily rhythm. Rev-erbα represses expression of tyrosine hydroxylase which partly affects the daily rhythm of dopamine release [69].

**Fig. 3. Differences in BDNF-mediated signaling pathways in astrocytes and**



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**neurons.** ***A***, Astrocytes predominantly express TrkB.T1 and BDNF-TrkB.T1-$p75^{NTR}$-ceramide mediated signaling is strong in light/rest phase increasing mitochondrial activity backed by CK2-Nrf2 activation. Ceramide also activates protein phosphatase-1 (PP1) which facilitates glycogen synthesis. During the late light/rest to early dark/active phase, $p75^{NTR}$ levels are expected to be decreased like Per2, while increased BDNF-TrkB.T1-$A_{2A}$R-mediated signaling activates cAMP/PKA axis which suppresses mitochondrial activity and increases glycogen hydrolysis [31]. ***B***, As neurons express both TrkB.FL and TrkB.T1, the signaling pathways differ from those in astrocytes. Notably, BDNF-TrkB.FL-$p75^{NTR}$ signaling inhibits ceramide generation and induces temporal internalization of $p75^{NTR}$ in CD63-positive multivesicular bodies (MVBs) for exosomes [74]. Therefore, BDNF may not efficiently activate Nrf2 in neurons via BDNF-TrkB.T1-$p75^{NTR}$ signaling. But, local activation of ceramide-PP1 signaling in periphery may play a role in transport of cargos along axons in neurons.

**Fig. 4. BDNF secretion and processing in cultured hippocampal neurons and astrocytes.**

***A***, Activated hippocampal neurons secrete proBDNF (marked by ❶). Plasmin cleaves proBDNF to generate BDNF. Tissue-type plasminogen activator (tPA) is required to produce plasmin from plasminogen. Plasminogen activator inhibitor-1 (PAI-1) suppresses tPA activity. Astrocytes absorb proBDNF through $p75^{NTR}$ and processed to generate mature BDNF within cells. Stimulation of astrocytes with ATP or with high $K^+$ releases mature BDNF (marked by ❷). ***B***, Various stimulations open pannexin 1 channels in astrocytes and secrete ATP (see Table 2). Extracellular ATP stimulates

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P2X7R leading to induction of membrane trafficking to secrete mature BDNF and membrane microparticles including exosomes.

**Fig. 5. Daily increase in plasma glucocorticoids leads to opening Pannexin 1 hemichannels and promotes interaction between astrocytes and neurons.** ***A*****,** Secretion of adrenal stress hormone glucocorticoids (GCs) is under the strict control of the master clock and HPA neuroendocrine axis. GC/corticosterone in plasma peaks (~50 ng/mL) just before the initiation of dark/active phase in mice [123]. GC activates its receptor (GCR) which modulates expression of a wide variety of genes. A notable target of GCs-GCR is serum- and glucocorticoid-induced protein kinase-1 (Sgk-1), which mobilizes calcium signaling leading to opening pannexin 1 hemichannels in astrocytes. The peak of released ATP in the cerebrospinal fluid is about 0.5 nmol/mg protein [123]. ***B***, We propose that systematic material transfer from astrocytes to neurons occur via microparticles and exosomes during the phase change from the light/rest to dark/active phase. Released BDNF from astrocytes and intercellular transfer of materials facilitate rapid metabolic changes in both cells, resulting in protection of neurons against oxidative damage and in increase in nursing activity of astrocytes.

**Table 1. Differential Nrf2-ARE regulated genes in astrocytes and neurons.**

| **Increased transcripts in astrocytes (total 97 genes)** | **Increased transcripts in neurons (total 48 genes)** |
|---|---|
| **Glucose metabolism**: Glucose phosphate isomerase, Glyceraldehyde-3-phosphate DH, Enolase, Aconitase 2, Glucose transporter 1, Glucose-6-phosphate dehydrogenase, Glucan branching enzyme 1, etc. | **Mitochondria related**:<br>Mt creatine kinase,<br>NADH dehydrogenase (Ndufa1),<br>S100 calcium binding protein A1.<br>**Others**: |

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| | |
|---|---|
| **GSH metabolism**: GSTs (α4, mu 1, mu 3, pi 2, microsomal), Glutamate-cysteine-ligase. | Glucose phosphate isomerase 1, GSTs (α2, mu 1), |
| **Antioxidants and others**: Peroxiredoxin 1 & 6, Thioredoxin reductase 1, Catalase, NQO1, Ferritin light/heavy chain, Aldehyde dehydrogenase, Sequestosome-1 (p62/A170/ZIP), etc. | Vascular cell adhesion molecule, Synaptotagmin 4, GABA-A receptor, etc. |

Compiled from supplemental Tables 2 and 3 in Kraft et al.[33]. The authors prepared fresh astrocytes and neurons from brain of mouse embryos and stimulated with 10 μM tBHQ for 24 h, then mRNAs were analyzed.

**Table 2. Stimulations that open pannexin 1 channels in astrocytes**.

| Agents or conditions | Mechanisms | References |
|---|---|---|
| High potassium | High $K^+$ induces a shift of activation potentials to more physiological range | [89], [90] |
| Interaction with neurons | αvβ3 integrin (astrocytes) and Thy-1 (neuron) interaction | [107] |
| Glucocorticoids | Corticosterone activates SGK-1 in mice astrocytes | [121] |

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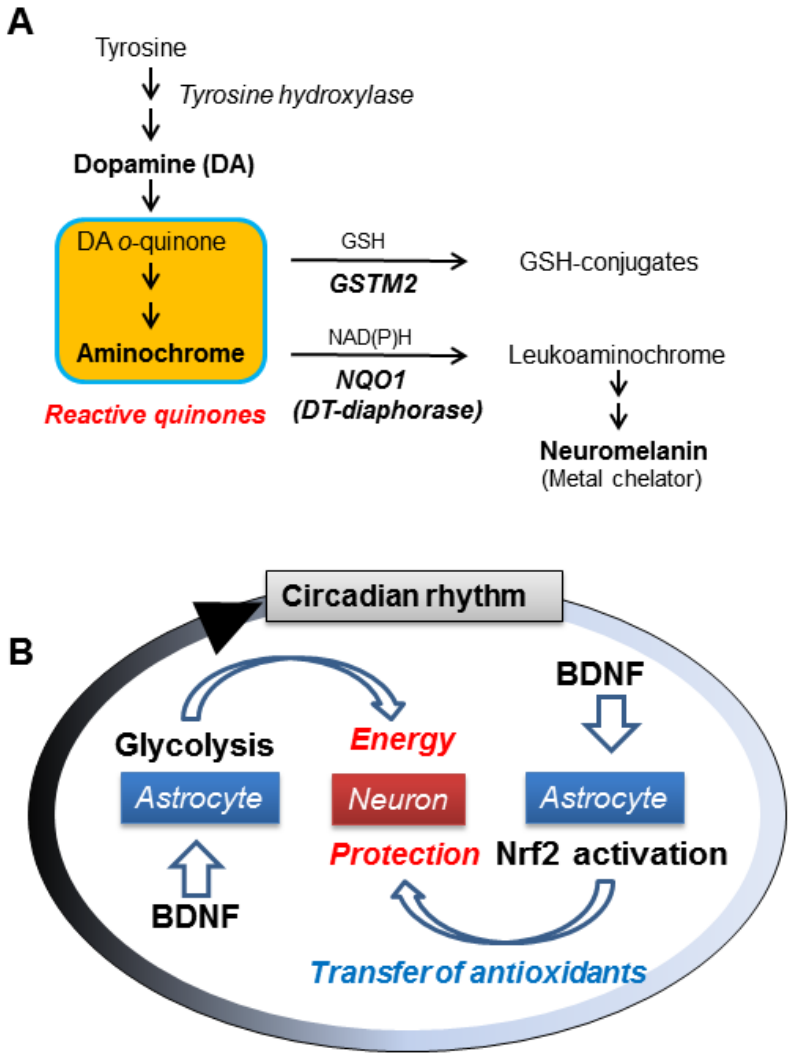
A
Tyrosine
Tyrosine hydroxylase
Dopamine (DA)
DA o-quinone
Aminochrome
Reactive quinones
GSH
GSTM2
GSH-conjugates
NAD(P)H
NQO1
(DT-diaphorase)
Leukoaminochrome
Neuromelanin
(Metal chelator)
B
Circadian rhythm
Glycolysis
Energy
BDNF
Astrocyte
Neuron
Astrocyte
Protection
Nrf2 activation
BDNF
Transfer of antioxidants

Fig. 1

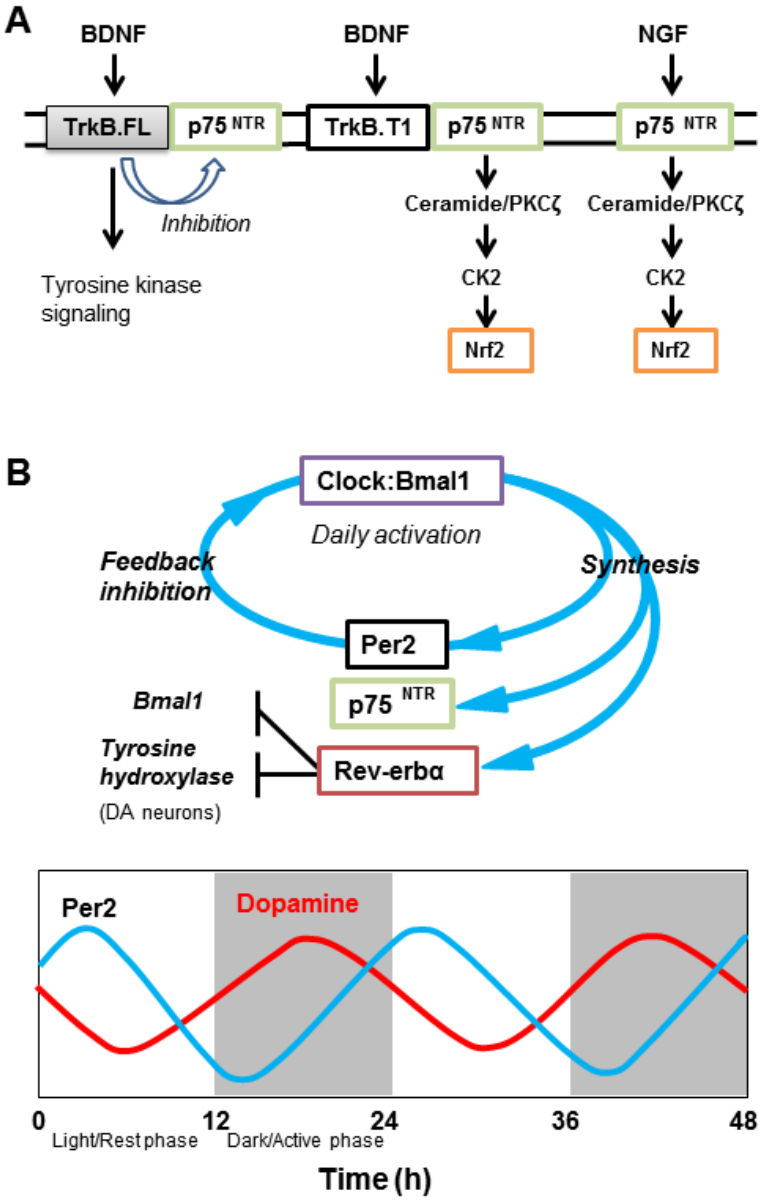
A
BDNF
BDNF
NGF
TrkB.FL
p75 NTR
TrkB.T1
p75 NTR
p75 NTR
Inhibition
Ceramide/PKCζ
Ceramide/PKCζ
Tyrosine kinase signaling
CK2
CK2
Nrf2
Nrf2
B
Clock:Bmal1
Daily activation
Feedback inhibition
Synthesis
Per2
Bmal1
p75 NTR
Tyrosine hydroxylase
(DA neurons)
Rev-erbα
Per2
Dopamine
0
12
24
36
48
Light/Rest phase
Dark/Active phase
Time (h)

Fig. 2

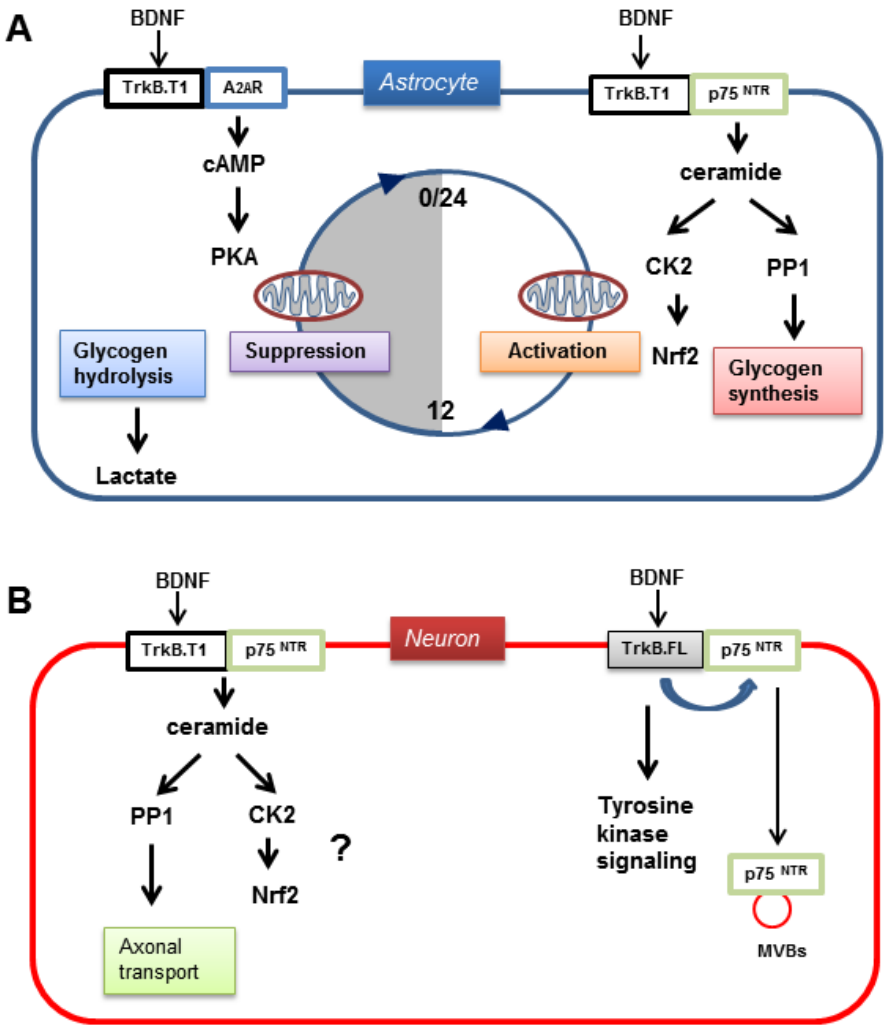
A
BDNF
TrkB.T1
A2AR
Astrocyte
BDNF
TrkB.T1
p75 NTR
cAMP
PKA
ceramide
CK2
PP1
0/24
Glycogen hydrolysis
Suppression
Activation
Nrf2
Glycogen synthesis
12
Lactate
B
BDNF
TrkB.T1
p75 NTR
Neuron
BDNF
TrkB.FL
p75 NTR
ceramide
PP1
CK2
?
Nrf2
Tyrosine kinase signaling
p75 NTR
Axonal transport
MVBs

Fig. 3

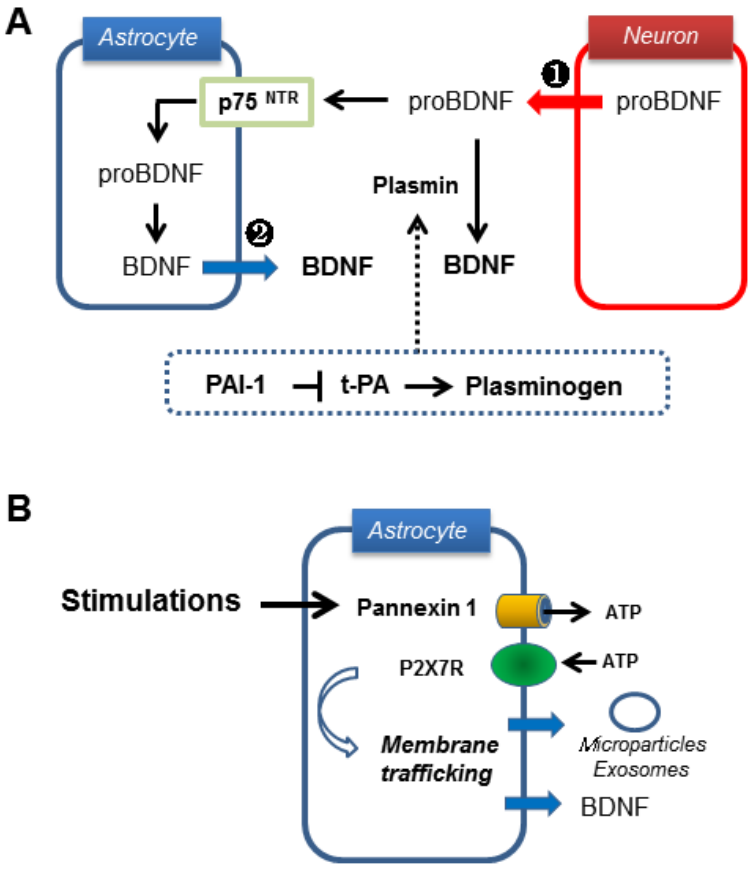
A
Astrocyte
Neuron
p75 NTR
proBDNF
proBDNF
proBDNF
proBDNF
Plasmin
BDNF
BDNF
BDNF
PAI-1
t-PA
Plasminogen
B
Astrocyte
Stimulations
Pannexin 1
ATP
P2X7R
ATP
Membrane trafficking
Microparticles Exosomes
BDNF

Fig. 4

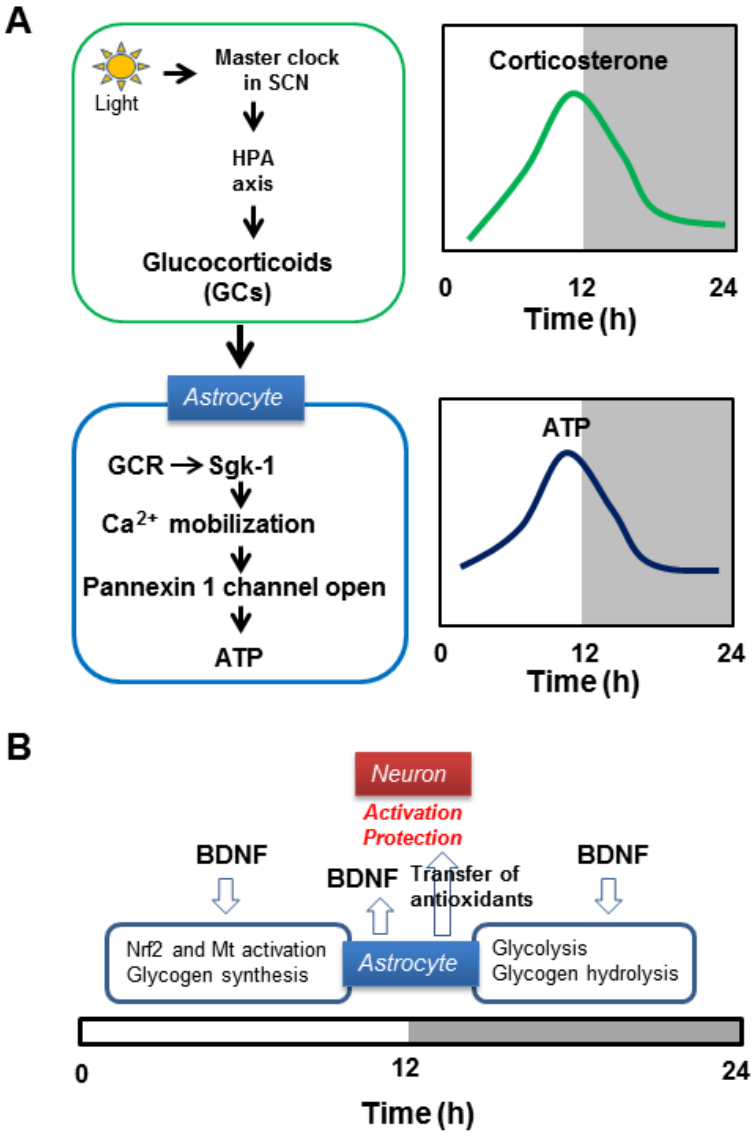
A
Light
Master clock in SCN
HPA axis
Glucocorticoids (GCs)
Corticosterone
0
12
24
Time (h)
Astrocyte
GCR → Sgk-1
Ca2+ mobilization
Pannexin 1 channel open
ATP
ATP
0
12
24
Time (h)
B
Neuron
Activation
Protection
BDNF
BDNF
Transfer of antioxidants
BDNF
Nrf2 and Mt activation
Glycogen synthesis
Astrocyte
Glycolysis
Glycogen hydrolysis
0
12
24
Time (h)

Fig. 5

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Highlight

- Astrocytes protect neurons from ferroptosis-like death
- BDNF-TrkB.T1-$p75^{NTR}$ axis activates Nrf2 in astrocytes
- Astrocytes transfer antioxidants to neurons via exosomes
- Pannexin 1-P2X7R axis in astrocytes facilitates secretion of exosomes and BDNF
- Glucocorticoids open pannexin 1 channels releasing ATP from astrocytes

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