Review

Inhibition of GTRAP3-18 May Increase Neuroprotective Glutathione (GSH) Synthesis

Koji Aoyama and Toshio Nakaki *

Department of Pharmacology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi, Tokyo 173-8605, Japan; E-Mail: kaoyama@med.teikyo-u.ac.jp

* Author to whom correspondence should be addressed; E-Mail: nakaki@med.teikyo-u.ac.jp; Tel.: +81-3-3964-1211; Fax: +81-3-3964-0602.

Abstract: Glutathione (GSH) is a tripeptide consisting of glutamate, cysteine, and glycine; it has a variety of functions in the central nervous system. Brain GSH depletion is considered a preclinical sign in age-related neurodegenerative diseases, and it promotes the subsequent processes toward neurotoxicity. A neuroprotective mechanism accomplished by increasing GSH synthesis could be a promising approach in the treatment of neurodegenerative diseases. In neurons, cysteine is the rate-limiting substrate for GSH synthesis. Excitatory amino acid carrier 1 (EAAC1) is a neuronal cysteine/glutamate transporter in the brain. EAAC1 translocation to the plasma membrane promotes cysteine uptake, leading to GSH synthesis, while being negatively regulated by glutamate transport associated protein 3-18 (GTRAP3-18). Our recent studies have suggested GTRAP3-18 as an inhibitory factor for neuronal GSH synthesis. Inhibiting GTRAP3-18 function is an endogenous mechanism to increase neuron-specific GSH synthesis in the brain. This review gives an overview of EAAC1-mediated GSH synthesis, and its regulatory mechanisms by GTRAP3-18 in the brain, and a potential approach against neurodegeneration.

1. Introduction

The brain is vulnerable to oxidative stress because of its high demand for oxygen, abundant unsaturated fatty acids that are targets of lipid peroxidation, and lower antioxidant enzyme activities

compared to other organs [1,2]. Under normal physiological conditions, antioxidant mechanisms function efficiently in the brain to overwhelm the lethal insults to neurons by reactive oxygen species (ROS). However, disequilibrium due to increased ROS production or decreased antioxidant defense systems would cause oxidative stress, which is considered a causal factor for neurodegeneration [3–5]. Glutathione (GSH) is the most abundant thiol-containing molecule and one of the most important substances in the brain for neuroprotection. GSH is a tripeptide synthesized from glutamate, cysteine and glycine via two enzymatic steps. Previous studies focused on the enzymatic regulation of GSH synthesis, and the regulatory mechanisms for the neuronal transport system of the rate-limiting substrate, cysteine, have not been clarified. Our recent studies have helped reveal the regulatory mechanisms for cysteine uptake leading to neuronal GSH synthesis. In the present review, we provide an overview of the key molecular mechanisms underlying cysteine uptake leading to increase neuronal GSH content in the brain.

2. Glutathione as an Antioxidant

GSH reacts non-enzymatically with superoxide, nitric oxide, hydroxyl radical, and peroxynitrite as an antioxidant [6] (Figure 1). Superoxide is generated by mitochondria in the process of ATP production. Superoxide or nitric oxide *per se* are not toxic unless they react with each other to form peroxynitrite [7], which is a potent oxidant in the brain [8]. Hydroxyl radicals form another potent oxidant, produced from hydrogen peroxide (H_2O_2) via the Fenton reaction or peroxynitrite decomposition [7,9], although the reaction rate of hydroxyl radical production is slow and the diffusion distance of hydroxyl radical is limited to much less than that of peroxynitrite [10]. GSH reacts directly with these oxidants to inhibit oxidative stress in the cell. In addition, GSH reacts enzymatically with GSH peroxidase (GPx) and GSH-*S*-transferase (GST) against neurodegeneration [6]. GPx requires GSH as electron donor to react with H_2O_2, which is produced from superoxide catalyzed by superoxide dismutase, or endogenous hydroperoxides, which are formed by lipid peroxidation [11]. In the process of peroxide disposal, GSH is oxidized to GSH disulfide (GSSG), which is then reduced back to GSH by GSH reductase (GR) with NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) [12]. In neurons, GR is sufficiently active to allow the quick reduction of the accumulated GSSG [13]. GST reacts with various xenobiotics to form GSH conjugation, leading to detoxication of the compounds and their excretion from the cell.

GSH is resistant to intracellular degradation; it is hydrolyzed solely by γ-glutamyltranspeptidase extracellularly to form γ-glutamyl moiety and a cysteinyl-glycine conjugate, which is then cleaved by dipeptidase to generate cysteine and glycine. GSH conjugation mediated by GST is a major cause of intracellular GSH depletion. Another cause of intracellular GSH depletion is due to thiol-disulfide formation from two GSH molecules to GSSG. Under normal conditions, the intracellular GSSG level is low, and the GSH/GSSG ratio in tissues is high (99:1) [14]. Under insulted conditions, the GSH/GSSG ratio would be 49:1 [15]. Under oxidative stress, GR maintains the equilibrium of the GSH/GSSG redox state in the cell. GSH can also reversibly react with protein thiol groups, which are important for the protein functions as enzymes or receptors, to prevent irreversible protein oxidation [16]. Severe oxidative stress leads to the excessive production of GSSG, overwhelming the reduction of GSSG back to GSH. The excessive GSSG is then exported outside the cell to decrease the

intracellular GSH level. Importantly, GSH depletion induced by oxidative stress can subsequently exacerbate oxidative injury in the brain. However, it has been debated whether oxidative stress consistently precedes GSH depletion in patients with neurodegenerative diseases.

Figure 1. Glutathione (GSH) as an antioxidant. Hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH) are degraded by GSH peroxidase (GPx) to water and alcohols, respectively. GSH disulfide (GSSG), the oxidized form of GSH, is reduced back to GSH by the reaction of GSH reductase (GR) with NADPH. Catalase can remove H_2O_2 but not ROOH under normal physiological conditions. GSH conjugates with various endogenous and xenobiotic compounds (X), mediated by GSH-*S*-transferase (GST) to remove X from the cell. GSH can also react non-enzymatically with superoxide ($O_2^{\bullet-}$), nitric oxide (NO), hydroxyl radical ($^{\bullet}OH$), and peroxynitrite ($ONOO^-$).
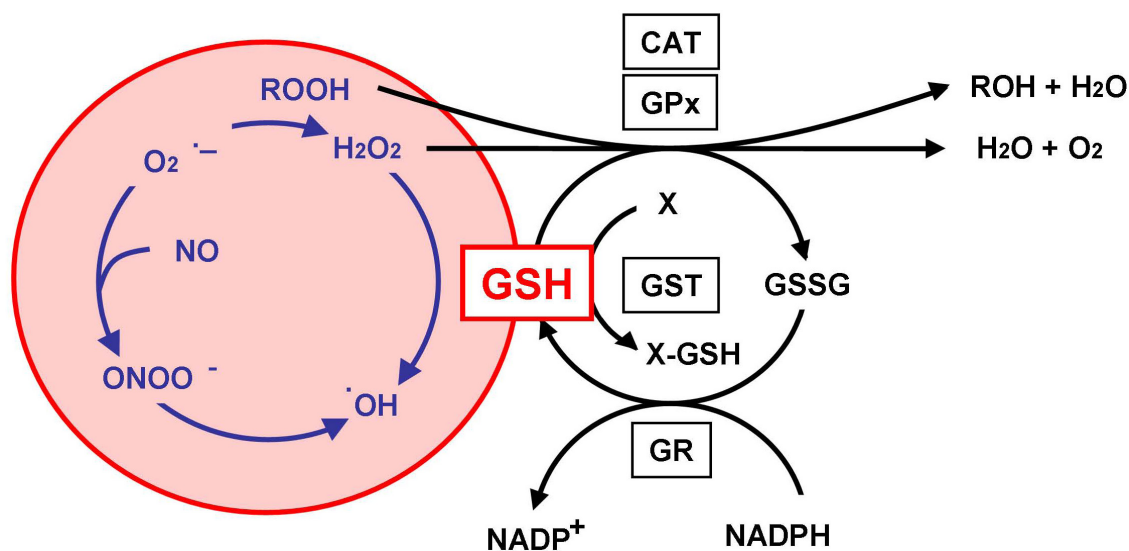
3. Glutathione Depletion and Neurodegeneration

The GSH level in the brain is approx. 2–3 mM [17]. However, the GSH content varies among brain regions and cell types. The GSH level is the highest in the cortex, followed by the cerebellum, hippocampus, and striatum, and it is lowest in the substantia nigra [18]. Importantly, the basal GSH level is lower in neurons than in glial cells [19], suggesting different mechanisms for preserving GSH homeostasis between neuron and glial cells. In addition, GPx activity is lower in neurons than in glial cells [13,19,20]. Certainly, the ability to detoxify peroxides is less efficient in neurons than in astrocytes [13,19]. Catalase, as is the case for GPx, can also detoxify H_2O_2 via degradation to H_2O and O_2. However, neuronal catalase activity does not compensate for the peroxide detoxication [19]. Catalase can remove only H_2O_2, but not hydroperoxides [21]. These results indicate that neurons are more vulnerable to oxidative stress than are glial cells in the brain, leading to neurodegeneration.

Previous articles indicate an involvement of GSH depletion in neurodegeneration [2,22–24]. Indeed, some neurodegenerative diseases have shown decreased GSH levels in the brain [25,26], but it remains unclear whether the decreased GSH level is a cause or an outcome of neurodegeneration. In patients with Parkinson's disease (PD), the GSH level was found to be significantly lower than that of age-matched controls [25], whereas the levels of other antioxidants, such as ascorbate and

α-tocopherol, were unchanged in the substantia nigra [27] and basal ganglia [28], respectively. Postmortem analysis revealed that normal individuals with incidental Lewy bodies—who would be considered pre-symptomatic PD subjects—showed decreased GSH levels in the substantia nigra compared to those of age-matched controls without Lewy bodies [29]. However, these pre-symptomatic PD subjects did not show any difference in the levels of pro-oxidants, such as iron, ferritin, copper, manganese, and zinc, or mitochondrial enzyme activities in the substantia nigra. These results strongly suggest that GSH depletion is the primary cause of neurodegeneration, and not only a result of oxidative stress [30,31].

4. Approaches to Increase the GSH Levels in the Brain

4.1. Cysteine Uptake

GSH synthesis consists of two consecutive enzymatic steps requiring ATP (Figure 2). The first step is the reaction of glutamate with cysteine catalyzed by glutamate cysteine ligase (GCL), and the second is the reaction of γ-glutamylcysteine with glycine catalyzed by GSH synthase (GS). In neurons, the availability of cysteine, but not that of glutamate or glycine, limits intracellular GSH levels [32]. Compared to the intracellular concentrations of glutamate and glycine, the cysteine concentration is maintained at much lower levels because of its toxicity to the cell [33–36]. The first step, *i.e.*, the catalysis by GCL, is considered the rate-limiting reaction for GSH synthesis. The Km value of GCL for cysteine is 0.1–0.3 mM: that of glutamate is 1.8 mM [37]. The intracellular glutamate concentration is much higher than the Km value, whereas the intracellular cysteine level is around the Km value. Therefore, the intracellular cysteine level is considered the rate-limiting precursor for GSH synthesis.

4.2. EAAC1 for Neuronal Cysteine Uptake

In neurons, cysteine uptake is mediated mainly by one of the Na^+-dependent excitatory amino acid transporters, named excitatory amino acid carrier 1 (EAAC1) (Figure 2). EAAC1 is present in both glutamatergic and GABAergic neurons [38]. A number of studies have demonstrated that oxidative stress is involved in glutamate-induced neurotoxicity. Free radicals can increase the glutamate level in the synaptic cleft by increasing glutamate release and decreasing glutamate re-uptake [39]. Neuronal NMDA receptor activation leads to the formation of superoxide, causing oxidative stress [40]. EAAC1 was first reported as a neuronal glutamate transporter, although most of the glutamate uptake in the brain is dependent on transport by the glial transporters, such as GLAST and GLT-1 [41–43].

Notably, EAAC1 can also transport cysteine, the rate-limiting substrate for GSH synthesis, at a rate comparable to that of glutamate, whereas GLAST and GLT-1 can preferentially transport glutamate [44]. A previous study demonstrated that the glutamate transport was abolished in the R447C mutant of EAAC1, whereas that of cysteine was conserved, suggesting a different cysteine transport system from that of glutamate [45]. A recent study showed that the [3H]glutamate uptake activity in proteoliposomes prepared from GLT-1 knockout mouse brain was quite low (~2% of wild-type) compared to that from the wild-type, suggesting that the contribution of EAAC1 to synaptic glutamate clearance is almost negligible [38]. Indeed, the EAAC1 density in the rat hippocampus was 100 times lower than that of GLT-1 [38]. EAAC1 is observed in neuronal soma and dendrites, but not in

synaptic terminals [38,46,47]. These results support the hypothesis that the main role of EAAC1 in the brain is not neurotransmission but neuronal metabolism related to cysteine uptake.

Figure 2. EAAC1/GTRAP3-18-mediated GSH synthesis in neurons. GSH is produced from three amino acids, *i.e.*, glutamate (Glu), cysteine (Cys), and glycine (Gly) by reactions with two enzymes, γ-glutamylcysteine ligase (GCL) and GSH synthase (GS). Translocated to the plasma membrane, EAAC1 transports glutamate and cysteine into the neuron to increase GSH synthesis. Glutamate transporters, as the functioning forms, are present predominantly as trimers on the plasma membrane. SNAP-23 facilitates EAAC1 expression on the plasma membrane. EAAC1 is subject to internalization into early endosome and is recycled back to the plasma membrane in a Rab11-dependent manner. Rab1A regulates multiple membrane trafficking pathways. GTRAP3-18, a protein in the endoplasmic reticulum (ER), binds to Rab1A to interfere with the ER-Golgi transport of EAAC1. Moreover, GTRAP3-18 directly interacts with EAAC1 and retains EAAC1 at the ER to inhibit neuronal GSH synthesis. Arl6ip1 is a GTRAP3-18 interacting protein leading to decrease in GTRAP3-18/EAAC1 interaction. Arl6ip1 positively modulates EAAC1-mediated glutamate transport.
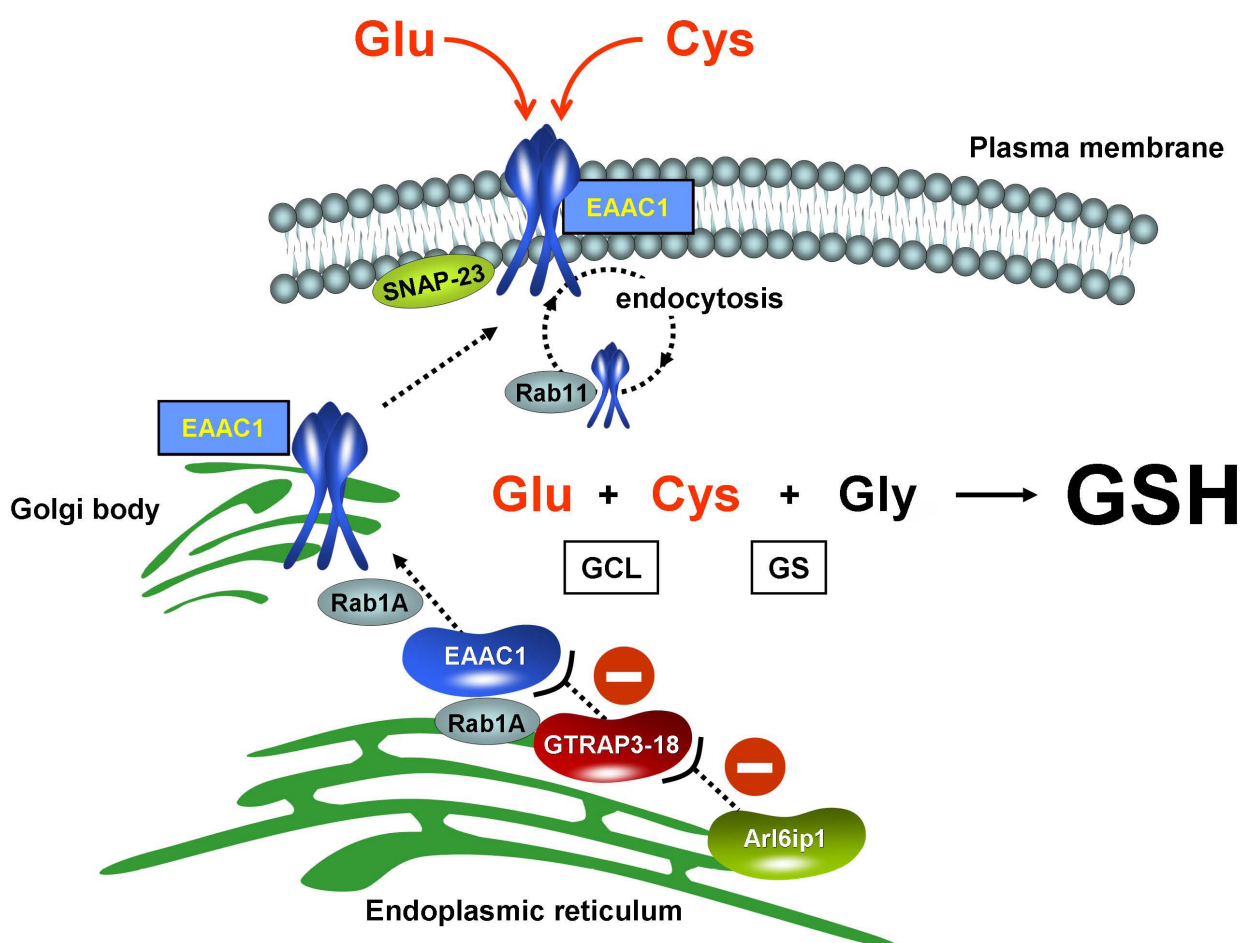
4.3. Regulation of EAAC1 Translocation to the Plasma Membrane

The expression of EAAC1 on the plasma membrane is only 20% of the total intracellular EAAC1 under normal conditions [48]. Glutamate transporters, including EAAC1, are predominantly present as trimers on the plasma membrane [42]. Translocated to the plasma membrane, EAAC1 can transport glutamate and cysteine for GSH synthesis. EAAC1 is translocated to the plasma membrane by phosphoinositide 3-kinase or protein kinase C (PKC) activation. Although EAAC1 is subject to a transcriptional regulation by nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant responsive element (ARE) pathway under oxidative stress [49], EAAC1 transport activity depends mainly on its cell surface expression, rather than *de novo* synthesis of the protein [48,50]. In the process of exocytosis, soluble *N*-ethylmaleimide-sensitive attachment protein receptor (SNARE) proteins facilitate the fusion of secretory vesicles to the plasma membrane. SNAP-23, a member of the SNARE family in the plasma membrane, is involved in EAAC1 trafficking to the plasma membrane [51]. The half-life of EAAC1 expression on the plasma membrane is approx. 5–7 min [48]. A substantial intracellular pool of EAAC1 is accomplished by endocytosis. Constitutive EAAC1 trafficking from endosome relies on a small GTP binding protein (G protein) activity. Small Rab GTPases regulate intracellular vesicle trafficking, especially in neurons [52,53]. These small G proteins are localized at the cytoplasmic faces of distinct membrane compartments. Recent studies demonstrated that EAAC1 was recycled back from endosomes to the plasma membrane in a Rab11-dependent manner [54] and that Rab11 dysfunction slowed EAAC1 trafficking to the cell surface, leading to impairments of both cysteine uptake and GSH synthesis [55].

4.4. GTRAP3-18

EAAC1 expression on the plasma membrane is negatively regulated by glutamate transport associated protein 3-18 (GTRAP3-18). By a yeast two-hybrid screen system from rat brain, GTRAP3-18 was isolated as an EAAC1-interacting protein [56]. GTRAP3-18 can specifically interact with EAAC1, but not with the other types of glutamate transporters. GTRAP3-18 is a member of the prenylated Rab acceptor (PRA) family [57]. There are two isoforms, PRA1 and PRA2, showing intracellular localization to the Golgi complex and the endoplasmic reticulum (ER), respectively [57]. Mouse GTRAP3-18 is almost identical to rat PRA2 [57,58]. GTRAP3-18/PRA2 is a 188-amino-acid protein and localizes in the ER with two extensive hydrophobic domains spanning amino acids 47 to 82 and 101 to 135, respectively [57], which are tightly attached to the ER membrane with four transmembrane domains and cytosolic *N* and *C* termini [59]. GTRAP3-18 tends to be dimeric [56], and the oligomerization of the four transmembrane domains is crucial for its activity [59]. Similarly, the oligomeric nature of the Yip family (the PRA family in yeast) has been demonstrated to enhance the protein-binding activity and selectivity [60].

The homo-oligomerization of GTRAP3-18 molecules may enhance its activity and specificity to certain oligomeric protein complexes. The C-terminal of GTRAP3-18 has a cluster of basic residues and a weak coiled-coil formation, which mediates protein-protein interaction. Changing the basic residues to an acidic residue (glutamate) resulted in partial localization of GTRAP3-18 to the Golgi complex [57]. This finding suggests that the *C*-terminal domain of GTRAP3-18 determines the

intracellular localization to the ER. The hydrophobic domain interaction between GTRAP3-18 and EAAC1 may inhibit EAAC1's exit from the ER [59]. GTRAP3-18 can bind to the *C*-terminal domain of EAAC1 (the last 87 amino acids) [56]. It can keep EAAC1 in the ER to inhibit EAAC1-mediated glutamate uptake, and it was observed that the glutamate uptake was elevated in accord with the reduction of the GTRAP3-18 protein level [56]. GTRAP3-18 protein with truncation of the *C*-terminal domain was unable to reduce ER-Golgi trafficking of EAAC1, whereas *N*-terminal truncation of GTRAP3-18 retained this ability [59]. GTRAP3-18 contains two PKC phosphorylation sites as well as two putative cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II phosphorylation sites [58]. There are two serine residues (S18 and S30) and three tyrosine residues (Y42, Y47 and Y182) in mouse GTRAP3-18 sequences. GTRAP3-18 may be regulated by protein phosphorylation via intracellular signaling molecules.

ADP-ribosylation factor-like 6 interacting protein 1 (Arl6ip1) is a GTRAP3-18-associated protein to act as a positive modulator of EAAC1-mediated glutamate transport in a PKC activity-dependent manner [61] (Figure 2). Arl6ip1 can interact with the hydrophobic region (amino acids 103–117) of GTRAP3-18 and decrease the number of GTRAP3-18 molecules available for interacting with EAAC1 [61]. Arl6ip1 cannot directly interact with EAAC1; however, it indirectly promotes EAAC1-mediated glutamate transport activity. Arl6ip1 also has PKC phosphorylation motifs [61]. PKC activation might increase GTRAP3-18/Arl6ip1 interaction, while decreasing GTRAP3-18/EAAC1 interaction in neurons [61].

4.5. Possible Regulation of ER-Golgi Transport by GTRAP3-18

Protein prenylation on *C*-terminal cysteine (Cys) residues plays an important role in its molecular function. Most Rab proteins have either a Cys-x-Cys or Cys-Cys motif, of which both Cys residues are subject to prenylation (geranylgeranylation) [62]. This process is required not only for the membrane association but also for the protein-protein interaction [63,64]. GTRAP3-18 binds to Rab1A and Rab3A in either the GDP- (inactive) or GTP-bound (active) state, but the interaction was abolished when the Cys-Cys prenylation motif of Rab was deleted *in vitro* [57]. This result suggests that the prenylation might be needed for the interaction between GTRAP3-18 and these Rabs.

The function of Rab1A is to promote ER-Golgi transport of vesicles. A recent study demonstrated that GTRAP3-18, most likely due to its Rab1 inhibitory action, inhibits neurite growth *in vitro* [65]. Rab3A is the most abundant Rab protein, localized to synaptic vesicles [64] to play a role in the recruitment of synaptic vesicles for exocytosis. Rab3A plays a key regulatory role in Ca^{2+}-dependent exocytosis, particularly in neurotransmitter release from nerve terminals [66]. Although GTRAP3-18 is thought to be an ER protein, some reports have showed the subcellular localization of GTRAP3-18 in both the cytosol and the cell surface *in vitro* [56,67], suggesting a possible regulatory function for Rab3A.

4.6. Physiological Roles of GTRAP3-18

The physiological role of GTRAP3-18 is still poorly understood. The basal level of GTRAP3-18 is essentially low, and it is up-regulated by cell differentiation, heat shock, and oxidative stress [68–70]. Chronic administration of morphine to mice leads to a 300% to 400% increase in the GTRAP3-18 mRNA level in the amygdala [71]. Chronic morphine treatment also increased δ-opioid receptor

expression in the nucleus accumbens [72]. GTRAP3-18 knockdown significantly decreased the withdrawal response to chronic morphine treatment [72], although the precise mechanism of GTRAP3-18 in the withdrawal responses to morphine is still elusive. In addition, GTRAP3-18 is reported to be able to regulate the trafficking of other transporters and receptors, such as dopamine transporter, GABA transporter (GAT-1), β_2-adrenergic receptor, $\alpha_1\beta$ receptor, and dopamine D_2 receptor [59]. The common feature of these transporters and receptors is the formation of oligomeric complex before their exit from the ER. GTRAP3-18 may universally control neurotransmission by regulating the ER-Golgi transport of neuronal transporters and receptors.

4.7. Induction of GTRAP3-18 by Methyl-β-cyclodextrin

In the brain, GTRAP3-18 is widely expressed in the cerebral cortex, striatum, hippocampus and cerebellum [58]. GTRAP3-18 distribution in the brain showed widespread expression colocalized to neurons [56]. Strong GTRAP3-18 immunoreactivity was observed in the neuron-rich stratum pyramidale of the hippocampus and the Purkinje cells of the cerebellum [58], consistent with the distribution of EAAC1 [73–75]. In an *in vivo* study, the intraventricular administration of GTRAP3-18 antisense oligomers significantly increased cortical glutamate uptake by EAAC1 [56]. Methyl-β-cyclodextrin (MeβCD) significantly reduced Na^+-dependent EAAC1-mediated [^{3}H]glutamate uptake and increased GTRAP3-18 protein expression *in vitro* [76]. Intracerebroventricular administration of MeβCD to the mouse brain resulted in a significant increase in GTRAP3-18 immunoreactivity in the hippocampus and cerebral cortex [76].

Cholesterol is required for Na^+-dependent glutamate transport [77]. MeβCD has a high affinity for cholesterol and has been shown to promote the efflux of cholesterol from cells [78]. Cholesterol depletion by MeβCD has been shown to induce cAMP response element-mediated gene expression [79] and to increase the activity of the extracellular signal-related kinase (ERK) [80]. cAMP- and ERK-dependent transcription factors may be responsible for the MeβCD-induced increase in GTRAP3-18 expression.

4.8. Regulation of GSH Synthesis by GTRAP3-18

Our previous cell culture studies demonstrated that increased GTRAP3-18 expression by MeβCD decreased the intracellular GSH content and showed vulnerability to oxidative stress, whereas knockdown of GTRAP3-18 expression by antisense oligonucleotide increased the intracellular GSH content and showed resistance to oxidative stress [67,81]. Intracerebroventricular injection models with MeβCD and siRNA for GTRAP3-18 also showed that increased or decreased GTRAP3-18 expression resulted in decreased or increased GSH level in the hippocampus, respectively. GTRAP3-18 negatively regulates the neuronal GSH level by direct interaction with EAAC1, which mediates neuronal cysteine uptake [67,81,82]. Our results suggest that GTRAP3-18 is a potential target for increasing the neuronal GSH level.

4.9. GTRAP3-18-Deficient Mouse

To further investigate the potential regulatory mechanism(s) underlying the increase in the neuronal GSH level *in vivo*, we generated GTRAP3-18-deficient (GTRAP3-18−/−) mice using embryonic stem cell technology [82]. The distribution of genotypes was close to the expected Mendelian frequency. GTRAP3-18−/− mice showed neither hindlimb clasping behavior, which is observed in neurodegenerative mice, nor histological abnormality in the brain. A recent study showed that the expression of GTRAP3-18 significantly declined from the embryonic stage to post-natal stage in the rat brains [65]. In addition, *in vitro* studies demonstrated that overexpression of GTRAP3-18 inhibited neurite outgrowth by blocking Rab1 function, whereas the knockdown of GTRAP3-18 had no effect on neurite length or the cell viability [65]. These results suggest a minor influence of the deletion of *GTRAP3-18* on neurodevelopment, at least morphologically.

The GTRAP3-18−/− mice showed an increased expression of EAAC1 on the plasma membrane [82]. EAAC1 activity is thought to largely depend on its cell surface expression, and not on *de novo* protein synthesis. The total amounts of brain glutamate, glycine, and GABA did not differ significantly between GTRAP3-18−/− and wild-type mice, but those of cysteine and GSH in the brain were significantly higher in the GTRAP3-18−/− mice compared to the wild-type mice. These results indicate that the ability of EAAC1 to increase cysteine uptake for GSH synthesis is potentiated in GTRAP3-18−/− mice. An immunostaining analysis showed that the increased GSH level was derived from neurons, but not from astrocytes or microglias [82]. Specifically, brain slices from GTRAP3-18−/− mice were resistant to oxidative stress. These results suggest that inhibition of GTRAP3-18 function leads to neuroprotection by increasing neuronal GSH synthesis.

Previous studies demonstrated that EAAC1 expression was increased in the rat hippocampus during the Morris water maze [83] and contextual fear conditioning tests, or by induction of long-term potentiation (LTP) in the CA1 region [84], while it was reported that EAAC1−/− mice showed impaired learning and memory [85,86]. Glutamate uptake is involved in memory formation [87–89]. Glial glutamate transporters can affect the duration and amplitude of excitatory postsynaptic potentials and currents by glutamate uptake [90], whereas the contribution of EAAC1 to the synaptic clearance of glutamate seems to be negligible [38]. Thus, it is unlikely that increased EAAC1 expression on the plasma membrane in GTRAP3-18−/− mice facilitates LTP induction by increasing the synaptic clearance of glutamate.

Another possible mechanism underlying the cognitive decline in EAAC1−/− mice is mediated by neuronal GSH depletion with aging. The presenile, but not young, EAAC1−/− mice showed brain atrophy and cognitive decline with aging compared to age-matched wild-type mice [85]. Neurons in the hippocampus of the EAAC1–/− mice showed low GSH and high oxidant levels, causing them to be vulnerable to oxidative stress. GSH depletion due to EAAC1 dysfunction could be the primary cause of age-dependent neurodegeneration. In our study [82], GTRAP3-18−/− mice showed high neuronal GSH levels and resistance to oxidative stress. They also showed better performance at forced motor/spatial learning and memory tests using rotarod and Morris water maze tests compared to age-matched wild-type mice. However, all of the mice in this study were under 6 months old, which is not old enough to develop age-dependent neurodegeneration. It is still unclear how GTRAP3-18 regulates the hippocampus-dependent learning process. The possibility that GTRAP3-18−/− mice

might have facilitated learning and memory functions due to mechanisms not related to EAAC1 cannot be excluded.

5. Antioxidant Supplementation

Brain accessible antioxidants may provide a potential approach to slow the onset and progression of neurodegenerative diseases. However, approaches for increasing the GSH level in the brain are a matter of dispute. At present, there is no exogenous antioxidant for clinical use to demonstrate a therapeutic effect against neurodegeneration.

5.1. L-cysteine and Glutathione

L-cysteine barely penetrates the blood–brain barrier (BBB) due to its lack of an acidic omega side chain [36]. GSH also does not cross the BBB easily, and it is rapidly oxidized into GSSG in blood [91–93]. The plasma half-life of intravenously administered GSH is approximate 2–3 min [93–95] and only 0.5% of injected GSH could pass the BBB *in vivo* [91]. Peripherally administered GSH does not seem to function as an antioxidant in the brain.

5.2. Vitamins

Ascorbate (Vitamin C) and α-tocopherol (Vitamin E) are also important antioxidants in the brain [96–98]. The concentration of ascorbate in the human brain ranges from 1 to 2.6 mM [99], similar to that the concentration of GSH in the brain. However, humans cannot produce ascorbate and the BBB almost completely blocks ascorbate penetration into the brain [100]. Alpha-tocopherol is the most potent antioxidant in the lipid part of the biological membrane [98]. However, the α-tocopherol level in the brain is relatively lower than those of ascorbate and GSH [17,98,99,101]. In addition, the oral administration of α-tocopherol did not increase its concentration in the central nervous system (CNS) due to its limited penetration of the BBB [102].

Indeed, supplementation with ascorbate, α-tocopherol, or both, have failed to produce conspicuous benefit in patients with neurodegenerative diseases such as PD and Alzheimer's disease (AD) [103–107], although several conflicting lines of evidence have been reported [108–110]. In addition, it was reported that the levels of ascorbate and α-tocopherol in the CNS did not change in patients with PD and AD, compared with controls [27,28,101,111]. The principal function of ascorbate and α-tocopherol as antioxidants may be based on a synergistic effect with GSH in the CNS [97,112].

5.3. Uric Acid

Uric acid (UA) is an important antioxidant in blood. UA scavenges singlet oxygen, hydroxyl radicals, and peroxynitrite in blood at its physiological concentration [113]. Indeed, epidemiological studies have shown an association between low plasma UA levels and neurodegenerative diseases [114]. However, the UA level is much lower in the CNS than in blood [115,116]. It is still unclear whether UA is able to act as a direct antioxidant in the brain. We recently reported that UA treatment increased both cysteine uptake *in vitro* and the brain GSH level at the physiological concentration *in vivo* [117]. Our results also showed that mouse hippocampal slices treated with UA were resistant to oxidative

stress and increased the GSH level in neurons. UA might indirectly exert a neuroprotective effect via the mediation of GSH synthesis in the brain by EAAC1, although further studies are needed to elucidate the mechanisms of any UA-mediated neuroprotective effect in the brain.

5.4. N-acetylcysteine

N-acetylcysteine (NAC) is a promising compound to increase GSH synthesis in the brain. NAC stimulates GSH synthesis not only by providing a source of cysteine, but also by activating GR [118]. NAC can permeate the cell membrane without the neuronal cysteine transporter EAAC1 [85,119] to yield cysteine via intracellular deacetylation. NAC also acts as a direct chemical antioxidant, although its potency is less than that of GSH [120]. Chronic dietary administration with 0.3% NAC containing food pellets reduced the protein carbonyl contents in synaptic mitochondria even without affecting the GSH level [121], although a single intraperitoneal injection of 150 mg/kg NAC can increase the GSH level in the mouse brain [85].

Indeed, neuroprotective effects of NAC have been reported on amyloid β [122], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [123], 6-hydroxydopamine [124], CNS trauma [125], and brain ischemia-induced neurotoxicity [119,126]. We also confirmed the neuroprotective ability of NAC to increase the GSH level in mesencephalic dopaminergic neurons [127]. However, the effect of NAC is not neuron-specific [128], and excessive production of cysteine may be neurotoxic [33–36]. The appropriate dose of NAC for clinical use against neurodegeneration remains to be determined.

6. Conclusions

In the treatment of neurodegenerative diseases, replacement therapy is still the main approach to ameliorate the symptoms, but not to inhibit neurodegeneration, whereas antioxidant therapy has been suggested to be a fundamental approach against neurodegeneration. Among the antioxidant agents, GSH plays a critical role in the CNS. Increasing the neuronal GSH content in the brain is a promising approach by which neuroprotective effects have been demonstrated. Not a small number of published papers have suggested potential candidates to increase neuronal GSH content. However, most of these candidates simply act as precursors for cysteine or GSH. Considering delivery systems into the brain, these exogenous supplementations do not seem to be the best way to increase the neuronal GSH level. We have focused on GTRAP3-18 as a target for neuroprotection. GTRAP3-18 is localized in neurons to negatively regulate EAAC1 function, a mechanism by which neurons can increase GSH synthesis. The endogenous regulation of GTRAP3-18 would be an alternative approach to the inhibition of neurodegeneration. However, considering a potential ability to regulate intracellular trafficking of various receptors and transporters, other, as yet unknown, physiological functions of GTRAP3-18 *in vivo* should be clarified.

References

1.   Ho, Y.S.; Magnenat, J.L.; Bronson, R.T.; Cao, J.; Gargano, M.; Sugawara, M.; Funk, C.D. Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *J. Biol. Chem.* **1997**, *272*, 16644–16651.

2. Dringen, R. Metabolism and functions of glutathione in brain. *Prog. Neurobiol.* **2000**, *62*, 649–671.

3. Coyle, J.T.; Puttfarcken, P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **1993**, *262*, 689–695.

4. Bains, J.S.; Shaw, C.A. Neurodegenerative disorders in humans: The role of glutathione in oxidative stress-mediated neuronal death. *Brain Res. Brain Res. Rev.* **1997**, *25*, 335–358.

5. Reed, T.T. Lipid peroxidation and neurodegenerative disease. *Free Radic. Biol. Med.* **2011**, *51*, 1302–1319.

6. Aoyama, K.; Watabe, M.; Nakaki, T. Regulation of neuronal glutathione synthesis. *J. Pharmacol. Sci.* **2008**, *108*, 227–238.

7. Pacher, P.; Beckman, J.S.; Liaudet, L. Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* **2007**, *87*, 315–424.

8. Aoyama, K.; Matsubara, K.; Fujikawa, Y.; Nagahiro, Y.; Shimizu, K.; Umegae, N.; Hayase, N.; Shiono, H.; Kobayashi, S. Nitration of manganese superoxide dismutase in cerebrospinal fluids is a marker for peroxynitrite-mediated oxidative stress in neurodegenerative diseases. *Ann. Neurol.* **2000**, *47*, 524–527.

9. Szabo, C.; Ischiropoulos, H.; Radi, R. Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics. *Nat. Rev. Drug Discov.* **2007**, *6*, 662–680.

10. Beckman, J.S. Peroxynitrite versus hydroxyl radical: The role of nitric oxide in superoxide-dependent cerebral injury. *Ann. N. Y. Acad. Sci.* **1994**, *738*, 69–75.

11. Kuhn, H.; Borchert, A. Regulation of enzymatic lipid peroxidation: The interplay of peroxidizing and peroxide reducing enzymes. *Free Radic. Biol. Med.* **2002**, *33*, 154–172.

12. Dringen, R.; Gutterer, J.M. Glutathione reductase from bovine brain. *Methods Enzymol.* **2002**, *348*, 281–288.

13. Dringen, R.; Kussmaul, L.; Gutterer, J.M.; Hirrlinger, J.; Hamprecht, B. The glutathione system of peroxide detoxification is less efficient in neurons than in astroglial cells. *J. Neurochem.* **1999**, *72*, 2523–2530.

14. Meister, A.; Anderson, M.E. Glutathione. *Annu. Rev. Biochem.* **1983**, *52*, 711–760.

15. Maher, P. The effects of stress and aging on glutathione metabolism. *Ageing Res. Rev.* **2005**, *4*, 288–314.

16. Giustarini, D.; Rossi, R.; Milzani, A.; Colombo, R.; Dalle-Donne, I. *S*-glutathionylation: From redox regulation of protein functions to human diseases. *J. Cell. Mol. Med.* **2004**, *8*, 201–212.

17. Cooper, A.J.; Kristal, B.S. Multiple roles of glutathione in the central nervous system. *Biol. Chem.* **1997**, *378*, 793–802.

18. Kang, Y.; Viswanath, V.; Jha, N.; Qiao, X.; Mo, J.Q.; Andersen, J.K. Brain gamma-glutamyl cysteine synthetase (GCS) mRNA expression patterns correlate with regional-specific enzyme activities and glutathione levels. *J. Neurosci. Res.* **1999**, *58*, 436–441.

19. Dringen, R.; Pawlowski, P.G.; Hirrlinger, J. Peroxide detoxification by brain cells. *J. Neurosci. Res.* **2005**, *79*, 157–165.

20. Desagher, S.; Glowinski, J.; Premont, J. Astrocytes protect neurons from hydrogen peroxide toxicity. *J. Neurosci.* **1996**, *16*, 2553–2562.

21. Aebi, H. Catalase *in vitro*. *Methods Enzymol.* **1984**, *105*, 121–126.

22. Jain, A.; Martensson, J.; Stole, E.; Auld, P.A.; Meister, A. Glutathione deficiency leads to mitochondrial damage in brain. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 1913–1917.

23. Dringen, R.; Gutterer, J.M.; Hirrlinger, J. Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur. J. Biochem.* **2000**, *267*, 4912–4916.

24. Schulz, J.B.; Lindenau, J.; Seyfried, J.; Dichgans, J. Glutathione, oxidative stress and neurodegeneration. *Eur. J. Biochem.* **2000**, *267*, 4904–4911.

25. Sian, J.; Dexter, D.T.; Lees, A.J.; Daniel, S.; Agid, Y.; Javoy-Agid, F.; Jenner, P.; Marsden, C.D. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann. Neurol.* **1994**, *36*, 348–355.

26. Ramassamy, C.; Averill, D.; Beffert, U.; Theroux, L.; Lussier-Cacan, S.; Cohn, J.S.; Christen, Y.; Schoofs, A.; Davignon, J.; Poirier, J. Oxidative insults are associated with apolipoprotein E genotype in Alzheimer's disease brain. *Neurobiol. Dis.* **2000**, *7*, 23–37.

27. Riederer, P.; Sofic, E.; Rausch, W.D.; Schmidt, B.; Reynolds, G.P.; Jellinger, K.; Youdim, M.B. Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. *J. Neurochem.* **1989**, *52*, 515–520.

28. Dexter, D.T.; Ward, R.J.; Wells, F.R.; Daniel, S.E.; Lees, A.J.; Peters, T.J.; Jenner, P.; Marsden, C.D. Alpha-tocopherol levels in brain are not altered in Parkinson's disease. *Ann. Neurol.* **1992**, *32*, 591–593.

29. Dexter, D.T.; Sian, J.; Rose, S.; Hindmarsh, J.G.; Mann, V.M.; Cooper, J.M.; Wells, F.R.; Daniel, S.E.; Lees, A.J.; Schapira, A.H.; *et al.* Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. *Ann. Neurol.* **1994**, *35*, 38–44.

30. Jenner, P. Oxidative damage in neurodegenerative disease. *Lancet* **1994**, *344*, 796–798.

31. Jenner, P. Oxidative stress in Parkinson's disease. *Ann. Neurol.* **2003**, *53*, S26–S36; discussion S36–S28.

32. Dringen, R.; Pfeiffer, B.; Hamprecht, B. Synthesis of the antioxidant glutathione in neurons: Supply by astrocytes of CysGly as precursor for neuronal glutathione. *J. Neurosci.* **1999**, *19*, 562–569.

33. Puka-Sundvall, M.; Eriksson, P.; Nilsson, M.; Sandberg, M.; Lehmann, A. Neurotoxicity of cysteine: Interaction with glutamate. *Brain Res.* **1995**, *705*, 65–70.

34. Janaky, R.; Varga, V.; Hermann, A.; Saransaari, P.; Oja, S.S. Mechanisms of L-cysteine neurotoxicity. *Neurochem. Res.* **2000**, *25*, 1397–1405.

35. Gazit, V.; Ben-Abraham, R.; Pick, C.G.; Ben-Shlomo, I.; Katz, Y. Long-term neurobehavioral and histological damage in brain of mice induced by L-cysteine. *Pharmacol. Biochem. Behav.* **2003**, *75*, 795–799.

36. Gazit, V.; Ben-Abraham, R.; Coleman, R.; Weizman, A.; Katz, Y. Cysteine-induced hypoglycemic brain damage: An alternative mechanism to excitotoxicity. *Amino Acids* **2004**, *26*, 163–168.

37. Richman, P.G.; Meister, A. Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. *J. Biol. Chem.* **1975**, *250*, 1422–1426.

38. Holmseth, S.; Dehnes, Y.; Huang, Y.H.; Follin-Arbelet, V.V.; Grutle, N.J.; Mylonakou, M.N.; Plachez, C.; Zhou, Y.; Furness, D.N.; Bergles, D.E.; *et al.* The density of EAAC1 (EAAT3) glutamate transporters expressed by neurons in the mammalian CNS. *J. Neurosci.* **2012**, *32*, 6000–6013.

39. Volterra, A.; Trotti, D.; Tromba, C.; Floridi, S.; Racagni, G. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. *J. Neurosci.* **1994**, *14*, 2924–2932.

40. Brennan, A.M.; Suh, S.W.; Won, S.J.; Narasimhan, P.; Kauppinen, T.M.; Lee, H.; Edling, Y.; Chan, P.H.; Swanson, R.A. NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation. *Nat. Neurosci.* **2009**, *12*, 857–863.

41. Lehre, K.P.; Levy, L.M.; Ottersen, O.P.; Storm-Mathisen, J.; Danbolt, N.C. Differential expression of two glial glutamate transporters in the rat brain: Quantitative and immunocytochemical observations. *J. Neurosci.* **1995**, *15*, 1835–1853.

42. Haugeto, O.; Ullensvang, K.; Levy, L.M.; Chaudhry, F.A.; Honore, T.; Nielsen, M.; Lehre, K.P.; Danbolt, N.C. Brain glutamate transporter proteins form homomultimers. *J. Biol. Chem.* **1996**, *271*, 27715–27722.

43. Tanaka, K.; Watase, K.; Manabe, T.; Yamada, K.; Watanabe, M.; Takahashi, K.; Iwama, H.; Nishikawa, T.; Ichihara, N.; Kikuchi, T.; *et al*. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* **1997**, *276*, 1699–1702.

44. Zerangue, N.; Kavanaugh, M.P. Interaction of L-cysteine with a human excitatory amino acid transporter. *J. Physiol.* **1996**, *493*, 419–423.

45. Bendahan, A.; Armon, A.; Madani, N.; Kavanaugh, M.P.; Kanner, B.I. Arginine 447 plays a pivotal role in substrate interactions in a neuronal glutamate transporter. *J. Biol. Chem.* **2000**, *275*, 37436–37442.

46. Rothstein, J.D.; Martin, L.; Levey, A.I.; Dykes-Hoberg, M.; Jin, L.; Wu, D.; Nash, N.; Kuncl, R.W. Localization of neuronal and glial glutamate transporters. *Neuron* **1994**, *13*, 713–725.

47. Shashidharan, P.; Huntley, G.W.; Murray, J.M.; Buku, A.; Moran, T.; Walsh, M.J.; Morrison, J.H.; Plaitakis, A. Immunohistochemical localization of the neuron-specific glutamate transporter EAAC1 (EAAT3) in rat brain and spinal cord revealed by a novel monoclonal antibody. *Brain Res.* **1997**, *773*, 139–148.

48. Fournier, K.M.; Gonzalez, M.I.; Robinson, M.B. Rapid trafficking of the neuronal glutamate transporter, EAAC1: Evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor. *J. Biol. Chem.* **2004**, *279*, 34505–34513.

49. Escartin, C.; Won, S.J.; Malgorn, C.; Auregan, G.; Berman, A.E.; Chen, P.C.; Deglon, N.; Johnson, J.A.; Suh, S.W.; Swanson, R.A. Nuclear factor erythroid 2-related factor 2 facilitates neuronal glutathione synthesis by upregulating neuronal excitatory amino acid transporter 3 expression. *J. Neurosci.* **2011**, *31*, 7392–7401.

50. Davis, K.E.; Straff, D.J.; Weinstein, E.A.; Bannerman, P.G.; Correale, D.M.; Rothstein, J.D.; Robinson, M.B. Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. *J. Neurosci.* **1998**, *18*, 2475–2485.

51. Fournier, K.M.; Robinson, M.B. A dominant-negative variant of SNAP-23 decreases the cell surface expression of the neuronal glutamate transporter EAAC1 by slowing constitutive delivery. *Neurochem. Int.* **2006**, *48*, 596–603.

52. Zerial, M.; McBride, H. Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 107–117.

53. Fukuda, M. Regulation of secretory vesicle traffic by Rab small GTPases. *Cell. Mol. Life Sci.* **2008**, *65*, 2801–2813.

54. Gonzalez, M.I.; Susarla, B.T.; Fournier, K.M.; Sheldon, A.L.; Robinson, M.B. Constitutive endocytosis and recycling of the neuronal glutamate transporter, excitatory amino acid carrier 1. *J. Neurochem.* **2007**, *103*, 1917–1931.

55. Li, X.; Valencia, A.; Sapp, E.; Masso, N.; Alexander, J.; Reeves, P.; Kegel, K.B.; Aronin, N.; Difiglia, M. Aberrant Rab11-dependent trafficking of the neuronal glutamate transporter EAAC1 causes oxidative stress and cell death in Huntington's disease. *J. Neurosci.* **2010**, *30*, 4552–4561.

56. Lin, C.I.; Orlov, I.; Ruggiero, A.M.; Dykes-Hoberg, M.; Lee, A.; Jackson, M.; Rothstein, J.D. Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. *Nature* **2001**, *410*, 84–88.

57. Abdul-Ghani, M.; Gougeon, P.Y.; Prosser, D.C.; Da-Silva, L.F.; Ngsee, J.K. PRA isoforms are targeted to distinct membrane compartments. *J. Biol. Chem.* **2001**, *276*, 6225–6233.

58. Butchbach, M.E.; Lai, L.; Lin, C.L. Molecular cloning, gene structure, expression profile and functional characterization of the mouse glutamate transporter (EAAT3) interacting protein GTRAP3-18. *Gene* **2002**, *292*, 81–90.

59. Ruggiero, A.M.; Liu, Y.; Vidensky, S.; Maier, S.; Jung, E.; Farhan, H.; Robinson, M.B.; Sitte, H.H.; Rothstein, J.D. The endoplasmic reticulum exit of glutamate transporter is regulated by the inducible mammalian Yip6b/GTRAP3-18 protein. *J. Biol. Chem.* **2008**, *283*, 6175–6183.

60. Pfeffer, S.; Aivazian, D. Targeting Rab GTPases to distinct membrane compartments. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 886–896.

61. Akiduki, S.; Ikemoto, M.J. Modulation of the neural glutamate transporter EAAC1 by the addicsin-interacting protein ARL6IP1. *J. Biol. Chem.* **2008**, *283*, 31323–31332.

62. Takai, Y.; Sasaki, T.; Matozaki, T. Small GTP-binding proteins. *Physiol. Rev.* **2001**, *81*, 153–208.

63. Marshall, C.J. Protein prenylation: A mediator of protein-protein interactions. *Science* **1993**, *259*, 1865–1866.

64. Gonzalez, L., Jr.; Scheller, R.H. Regulation of membrane trafficking: Structural insights from a Rab/effector complex. *Cell* **1999**, *96*, 755–758.

65. Maier, S.; Reiterer, V.; Ruggiero, A.M.; Rothstein, J.D.; Thomas, S.; Dahm, R.; Sitte, H.H.; Farhan, H. GTRAP3-18 serves as a negative regulator of Rab1 in protein transport and neuronal differentiation. *J. Cell. Mol. Med.* **2009**, *13*, 114–124.

66. Takai, Y.; Sasaki, T.; Shirataki, H.; Nakanishi, H. Rab3A small GTP-binding protein in Ca(2+)-dependent exocytosis. *Genes Cells* **1996**, *1*, 615–632.

67. Watabe, M.; Aoyama, K.; Nakaki, T. Regulation of glutathione synthesis via interaction between glutamate transport-associated protein 3-18 (GTRAP3-18) and excitatory amino acid carrier-1 (EAAC1) at plasma membrane. *Mol. Pharmacol.* **2007**, *72*, 1103–1110.

68. Wang, N.P.; Zhou, J.W.; Li, A.P.; Cao, H.X.; Wang, X.R. The mechanism of JWA gene involved in oxidative stress of cells (in Chinese). *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* **2003**, *21*, 212–215.

69. Mao, W.G.; Li, A.P.; Ye, J.; Huang, S.; Li, A.Q.; Zhou, J.W. Expressions of JWA protein and heat stress protein 70 induced by cell differentiation inducers combined with heat stress in K562 cells (in Chinese). *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* **2004**, *22*, 60–63.

70. Chen, R.; Qiu, W.; Liu, Z.; Cao, X.; Zhu, T.; Li, A.; Wei, Q.; Zhou, J. Identification of JWA as a novel functional gene responsive to environmental oxidative stress induced by benzo[a]pyrene and hydrogen peroxide. *Free Radic. Biol. Med.* **2007**, *42*, 1704–1714.

71. Ikemoto, M.J.; Inoue, K.; Akiduki, S.; Osugi, T.; Imamura, T.; Ishida, N.; Ohtomi, M. Identification of addicsin/GTRAP3-18 as a chronic morphine-augmented gene in amygdala. *Neuroreport* **2002**, *13*, 2079–2084.

72. Wu, Y.; Chen, R.; Zhao, X.; Li, A.; Li, G.; Zhou, J. JWA regulates chronic morphine dependence via the delta opioid receptor. *Biochem. Biophys. Res. Commun.* **2011**, *409*, 520–525.

73. Furuta, A.; Martin, L.J.; Lin, C.L.; Dykes-Hoberg, M.; Rothstein, J.D. Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4. *Neuroscience* **1997**, *81*, 1031–1042.

74. Hediger, M.A.; Welbourne, T.C. Introduction: Glutamate transport, metabolism, and physiological responses. *Am. J. Physiol.* **1999**, *277*, F477–F480.

75. Hediger, M.A. Glutamate transporters in kidney and brain. *Am. J. Physiol.* **1999**, *277*, F487–492.

76. Butchbach, M.E.; Guo, H.; Lin, C.L. Methyl-beta-cyclodextrin but not retinoic acid reduces EAAT3-mediated glutamate uptake and increases GTRAP3-18 expression. *J. Neurochem.* **2003**, *84*, 891–894.

77. Shouffani, A.; Kanner, B.I. Cholesterol is required for the reconstruction of the sodium- and chloride-coupled, gamma-aminobutyric acid transporter from rat brain. *J. Biol. Chem.* **1990**, *265*, 6002–6008.

78. Kilsdonk, E.P.; Yancey, P.G.; Stoudt, G.W.; Bangerter, F.W.; Johnson, W.J.; Phillips, M.C.; Rothblat, G.H. Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **1995**, *270*, 17250–17256.

79. Middleton, A.; Nury, D.; Willington, S.J.; Latif, L.; Hill, S.J.; Middleton, B. Modulation by cellular cholesterol of gene transcription via the cyclic AMP response element. *Biochem. Pharmacol.* **2001**, *62*, 171–181.

80. Furuchi, T.; Anderson, R.G. Cholesterol depletion of caveolae causes hyperactivation of extracellular signal-related kinase (ERK). *J. Biol. Chem.* **1998**, *273*, 21099–21104.

81. Watabe, M.; Aoyama, K.; Nakaki, T. A dominant role of GTRAP3-18 in neuronal glutathione synthesis. *J. Neurosci.* **2008**, *28*, 9404–9413.

82. Aoyama, K.; Wang, F.; Matsumura, N.; Kiyonari, H.; Shioi, G.; Tanaka, K.; Kinoshita, C.; Kikuchi-Utsumi, K.; Watabe, M.; Nakaki, T. Increased neuronal glutathione and neuroprotection in GTRAP3-18-deficient mice. *Neurobiol. Dis.* **2012**, *45*, 973–982.

83. Fraticelli, A.I.; Thompson, K.J. EAAC1 protein expression is increased in the rat hippocampus during water maze learning. *Soc. Neurosci. Abstr.* **2011**, *37*, 342.313/E342.

84. Levenson, J.; Weeber, E.; Selcher, J.C.; Kategaya, L.S.; Sweatt, J.D.; Eskin, A. Long-term potentiation and contextual fear conditioning increase neuronal glutamate uptake. *Nat. Neurosci.* **2002**, *5*, 155–161.

85. Aoyama, K.; Suh, S.W.; Hamby, A.M.; Liu, J.; Chan, W.Y.; Chen, Y.; Swanson, R.A. Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. *Nat. Neurosci.* **2006**, *9*, 119–126.

86. Lee, S.; Park, S.H.; Zuo, Z. Effects of isoflurane on learning and memory functions of wild-type and glutamate transporter type 3 knockout mice. *J. Pharm. Pharmacol.* **2012**, *64*, 302–307.

87. Ng, K.T.; O'Dowd, B.S.; Rickard, N.S.; Robinson, S.R.; Gibbs, M.E.; Rainey, C.; Zhao, W.Q.; Sedman, G.L.; Hertz, L. Complex roles of glutamate in the Gibbs-Ng model of one-trial aversive learning in the new-born chick. *Neurosci. Biobehav. Rev.* **1997**, *21*, 45–54.

88. Levenson, J.; Endo, S.; Kategaya, L.S.; Fernandez, R.I.; Brabham, D.G.; Chin, J.; Byrne, J.H.; Eskin, A. Long-term regulation of neuronal high-affinity glutamate and glutamine uptake in Aplysia. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 12858–12863.

89. Maleszka, R.; Helliwell, P.; Kucharski, R. Pharmacological interference with glutamate re-uptake impairs long-term memory in the honeybee, apis mellifera. *Behav. Brain Res.* **2000**, *115*, 49–53.

90. Mennerick, S.; Zorumski, C.F. Glial contributions to excitatory neurotransmission in cultured hippocampal cells. *Nature* **1994**, *368*, 59–62.

91. Cornford, E.M.; Braun, L.D.; Crane, P.D.; Oldendorf, W.H. Blood-brain barrier restriction of peptides and the low uptake of enkephalins. *Endocrinology* **1978**, *103*, 1297–1303.

92. Lash, L.H.; Jones, D.P. Distribution of oxidized and reduced forms of glutathione and cysteine in rat plasma. *Arch. Biochem. Biophys.* **1985**, *240*, 583–592.

93. Ammon, H.P.; Melien, M.C.; Verspohl, E.J. Pharmacokinetics of intravenously administered glutathione in the rat. *J. Pharm. Pharmacol.* **1986**, *38*, 721–725.

94. Wendel, A.; Cikryt, P. The level and half-life of glutathione in human plasma. *FEBS Lett.* **1980**, *120*, 209–211.

95. Wendel, A.; Jaeschke, H. Drug-induced lipid peroxidation in mice—III. Glutathione content of liver, kidney and spleen after intravenous administration of free and liposomally entrapped glutathione. *Biochem. Pharmacol.* **1982**, *31*, 3607–3611.

96. Perry, T.L.; Yong, V.W.; Clavier, R.M.; Jones, K.; Wright, J.M.; Foulks, J.G.; Wall, R.A. Partial protection from the dopaminergic neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by four different antioxidants in the mouse. *Neurosci. Lett.* **1985**, *60*, 109–114.

97. Meister, A. Glutathione-ascorbic acid antioxidant system in animals. *J. Biol. Chem.* **1994**, *269*, 9397–9400.

98. Gilgun-Sherki, Y.; Melamed, E.; Offen, D. Oxidative stress induced-neurodegenerative diseases: The need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology* **2001**, *40*, 959–975.

99. Grunewald, R.A. Ascorbic acid in the brain. *Brain Res. Brain Res. Rev.* **1993**, *18*, 123–133.

100. Agus, D.B.; Gambhir, S.S.; Pardridge, W.M.; Spielholz, C.; Baselga, J.; Vera, J.C.; Golde, D.W. Vitamin C crosses the blood-brain barrier in the oxidized form through the glucose transporters. *J. Clin. Invest.* **1997**, *100*, 2842–2848.

101. Metcalfe, T.; Bowen, D.M.; Muller, D.P. Vitamin E concentrations in human brain of patients with Alzheimer's disease, fetuses with Down's syndrome, centenarians, and controls. *Neurochem. Res.* **1989**, *14*, 1209–1212.

102. Pappert, E.J.; Tangney, C.C.; Goetz, C.G.; Ling, Z.D.; Lipton, J.W.; Stebbins, G.T.; Carvey, P.M. Alpha-tocopherol in the ventricular cerebrospinal fluid of Parkinson's disease patients: Dose-response study and correlations with plasma levels. *Neurology* **1996**, *47*, 1037–1042.

103. Logroscino, G.; Marder, K.; Cote, L.; Tang, M.X.; Shea, S.; Mayeux, R. Dietary lipids and antioxidants in Parkinson's disease: A population-based, case-control study. *Ann. Neurol.* **1996**, *39*, 89–94.

104. Mendelsohn, A.B.; Belle, S.H.; Stoehr, G.P.; Ganguli, M. Use of antioxidant supplements and its association with cognitive function in a rural elderly cohort: The MoVIES Project. Monongahela Valley Independent Elders Survey. *Am. J. Epidemiol.* **1998**, *148*, 38–44.

105. Petersen, R.C.; Thomas, R.G.; Grundman, M.; Bennett, D.; Doody, R.; Ferris, S.; Galasko, D.; Jin, S.; Kaye, J.; Levey, A.; *et al*. Vitamin E and donepezil for the treatment of mild cognitive impairment. *N. Engl. J. Med.* **2005**, *352*, 2379–2388.

106. Isaac, M.G.; Quinn, R.; Tabet, N. Vitamin E for Alzheimer's disease and mild cognitive impairment. *Cochrane Database Syst. Rev.* **2008**, CD002854.

107. Galasko, D.R.; Peskind, E.; Clark, C.M.; Quinn, J.F.; Ringman, J.M.; Jicha, G.A.; Cotman, C.; Cottrell, B.; Montine, T.J.; Thomas, R.G.; *et al*. Antioxidants for Alzheimer Disease: A Randomized Clinical Trial With Cerebrospinal Fluid Biomarker Measures. *Arch. Neurol.* **2012**, *69*, 836–841.

108. Dexter, D.T.; Nanayakkara, I.; Goss-Sampson, M.A.; Muller, D.P.; Harding, A.E.; Marsden, C.D.; Jenner, P. Nigral dopaminergic cell loss in vitamin E deficient rats. *Neuroreport* **1994**, *5*, 1773–1776.

109. Sano, M.; Ernesto, C.; Thomas, R.G.; Klauber, M.R.; Schafer, K.; Grundman, M.; Woodbury, P.; Growdon, J.; Cotman, C.W.; Pfeiffer, E.; *et al*. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N. Engl. J. Med.* **1997**, *336*, 1216–1222.

110. Cristina Polidori, M.; De Spirt, S.; Stahl, W.; Pientka, L. Conflict of evidence: Carotenoids and other micronutrients in the prevention and treatment of cognitive impairment. *Biofactors* **2012**, *38*, 167–171.

111. Paraskevas, G.P.; Kapaki, E.; Libitaki, G.; Zournas, C.; Segditsa, I.; Papageorgiou, C. Ascorbate in healthy subjects, amyotrophic lateral sclerosis and Alzheimer's disease. *Acta. Neurol. Scand.* **1997**, *96*, 88–90.

112. Chen, H.; Tappel, A.L. Protection by vitamin E selenium, trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q, beta-carotene, canthaxanthin, and (+)-catechin against oxidative damage to liver slices measured by oxidized heme proteins. *Free Radic. Biol. Med.* **1994**, *16*, 437–444.

113. Ames, B.N.; Cathcart, R.; Schwiers, E.; Hochstein, P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6858–6862.

114. Kutzing, M.K.; Firestein, B.L. Altered uric acid levels and disease states. *J. Pharmacol. Exp. Ther.* **2008**, *324*, 1–7.

115. Hensley, K.; Maidt, M.L.; Yu, Z.; Sang, H.; Markesbery, W.R.; Floyd, R.A. Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. *J. Neurosci.* **1998**, *18*, 8126–8132.

116. Marklund, N.; Ostman, B.; Nalmo, L.; Persson, L.; Hillered, L. Hypoxanthine, uric acid and allantoin as indicators of in vivo free radical reactions. Description of a HPLC method and human brain microdialysis data. *Acta Neurochir. (Wien.)* **2000**, *142*, 1135–1141; discussion 1141–1132.

117. Aoyama, K.; Matsumura, N.; Watabe, M.; Wang, F.; Kikuchi-Utsumi, K.; Nakaki, T. Caffeine and uric acid mediate glutathione synthesis for neuroprotection. *Neuroscience* **2011**, *181*, 206–215.

118. De Flora, S.; Bennicelli, C.; Zanacchi, P.; Camoirano, A.; Morelli, A.; De Flora, A. *In vitro* effects of *N*-acetylcysteine on the mutagenicity of direct-acting compounds and procarcinogens. *Carcinogenesis* **1984**, *5*, 505–510.

119. Won, S.J.; Yoo, B.H.; Brennan, A.M.; Shin, B.S.; Kauppinen, T.M.; Berman, A.E.; Swanson, R.A.; Suh, S.W. EAAC1 gene deletion alters zinc homeostasis and exacerbates neuronal injury after transient cerebral ischemia. *J. Neurosci.* **2010**, *30*, 15409–15418.

120. Hussain, S.; Slikker, W., Jr.; Ali, S.F. Role of metallothionein and other antioxidants in scavenging superoxide radicals and their possible role in neuroprotection. *Neurochem. Int.* **1996**, *29*, 145–152.

121. Banaclocha, M.M.; Hernandez, A.I.; Martinez, N.; Ferrandiz, M.L. *N*-acetylcysteine protects against age-related increase in oxidized proteins in mouse synaptic mitochondria. *Brain Res.* **1997**, *762*, 256–258.

122. Fu, A.L.; Dong, Z.H.; Sun, M.J. Protective effect of *N*-acetyl-L-cysteine on amyloid beta-peptide-induced learning and memory deficits in mice. *Brain Res.* **2006**, *1109*, 201–206.

123. Park, S.W.; Kim, S.H.; Park, K.H.; Kim, S.D.; Kim, J.Y.; Baek, S.Y.; Chung, B.S.; Kang, C.D. Preventive effect of antioxidants in MPTP-induced mouse model of Parkinson's disease. *Neurosci. Lett.* **2004**, *363*, 243–246.

124. Munoz, A.M.; Rey, P.; Soto-Otero, R.; Guerra, M.J.; Labandeira-Garcia, J.L. Systemic administration of *N*-acetylcysteine protects dopaminergic neurons against 6-hydroxydopamine-induced degeneration. *J. Neurosci. Res.* **2004**, *76*, 551–562.

125. Hart, A.M.; Terenghi, G.; Kellerth, J.O.; Wiberg, M. Sensory neuroprotection, mitochondrial preservation, and therapeutic potential of *N*-acetyl-cysteine after nerve injury. *Neuroscience* **2004**, *125*, 91–101.

126. Sekhon, B.; Sekhon, C.; Khan, M.; Patel, S.J.; Singh, I.; Singh, A.K. *N*-Acetyl cysteine protects against injury in a rat model of focal cerebral ischemia. *Brain Res.* **2003**, *971*, 1–8.

127. Aoyama, K.; Matsumura, N.; Watabe, M.; Nakaki, T. Oxidative stress on EAAC1 is involved in MPTP-induced glutathione depletion and motor dysfunction. *Eur. J. Neurosci.* **2008**, *27*, 20–30.

128. Kranich, O.; Dringen, R.; Sandberg, M.; Hamprecht, B. Utilization of cysteine and cysteine precursors for the synthesis of glutathione in astroglial cultures: Preference for cystine. *Glia* **1998**, *22*, 11–18.