The Influence of GABA Metabolism on GABA Neurotransmission: The Role of Metabolic Regulatory Points and a Neuronal Glutamate Transporter

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Excessive excitatory drive in the brain is thought to underlie diseases such as epilepsy. One approach in the development of novel treatments for conditions characterized by hyper-excitability is the enhancement of GABA-mediated inhibition. While most current medical interventions target GABAergic neurotransmission postsynaptically (e.g. benzodiazepines, barbiturates), much less is known about potential presynaptic therapeutic targets at the GABAergic synapse. This review describes recent findings that have exemplified presynaptic mechanisms that may provide the basis for the development of novel treatments to alter inhibitory neurotransmission. GABA metabolism is summarized with an emphasis on the role of presynaptic regulatory points in GABA synthesis. In addition, the excitatory amino acid transporter 3 (EAAT3), which is thought to provide the substrate for GABA synthesis, will be described in detail. Finally, EAAT3 is presented as a potential therapeutic target to modulate GABA-mediated inhibition presynaptically, and the most recent findings on EAAT3's functional regulation by several key players are reviewed.

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GLUTAMATE AND GABA METABOLISM

Glutamate and GABA are the major excitatory and inhibitory neurotransmitters in the brain, respectively. Unlike other neurotransmitter systems, such as monoamines, reuptake and recycling of glutamate and GABA does not appear to be as important as new synthesis to replenish the pool of neurotransmitter for synaptic vesicle filling. Despite the critical importance of neurotransmitter supply, either to prevent depletion and maintain stable transmission or perhaps to dynamically adjust in response to demand, the metabolic pathways by which these transmitters are continuously supplied to synaptic terminals have not been resolved.

GABA is synthesized by the decarboxylation of glutamate, which is catalyzed by the enzyme glutamic acid decarboxylase (GAD). Neurons are not capable of synthesizing glutamate[1] on their own; therefore inhibitory neurons, like excitatory neurons, need a supply of glutamate. At least two possible pathways through which glutamate may be acquired exists: (1) the direct uptake of extracellular glutamate or (2) the uptake of glutamine, which can be converted to glutamate by neurons. Transporters serving both of these roles are expressed by GABAergic neurons, and both have been demonstrated to play roles in the synthesis of GABA[2-5].

GABA synthesis and synaptic vesicle filling are tightly coupled processes as revealed by biochemical assays. GAD65, the synaptically localized isoform of GAD, is associated with a complex of proteins on synaptic vesicles that includes the vesicular GABA transporter[6]. GABA synthesized from glutamate is taken up into synaptic vesicles preferentially over pre-existing GABA[7]. Electrophysiological studies demonstrated that inhibiting GAD results in a reduction in the size of miniature synaptic events, which represent the amount of GABA released from a single synaptic vesicle[8]. In contrast, knock-out of the predominant membrane transporter for GABA reuptake does not influence the size of these miniature events[9]. Taken together, these findings suggest that new synthesis is more important than recycling of existing GABA. Moreover, they demonstrate that any factors influencing GABA synthesis are likely to play an important role in maintaining, and possibly regulating, inhibitory synaptic transmission. Finally, GABA is catabolized by the action of GABA transaminase (GABA-T), which deaminates GABA to make succinic semialdehyde (SSA), and then SSA dehydrogenase (SSADH) converts SSA to succinate, which enters the TCA cycle. SSA can also be converted to γ–hydroxybutyrate (GBH) by the action of SSA reductase[10] (**Figure 1**).

HOW IS SUBSTRATE OBTAINED FOR PRODUCTION OF GLUTAMATE AND GABA?

Supply of substrate to inhibitory neurons for GABA synthesis is mediated by highly regulated, sodium-dependent solute transporters, which carry metabolites against their concentration gradients. EAAT3, a member of the high affinity glutamate transporter (excitatory amino acid transporter, EAAT) family, is expressed on somatodendritic

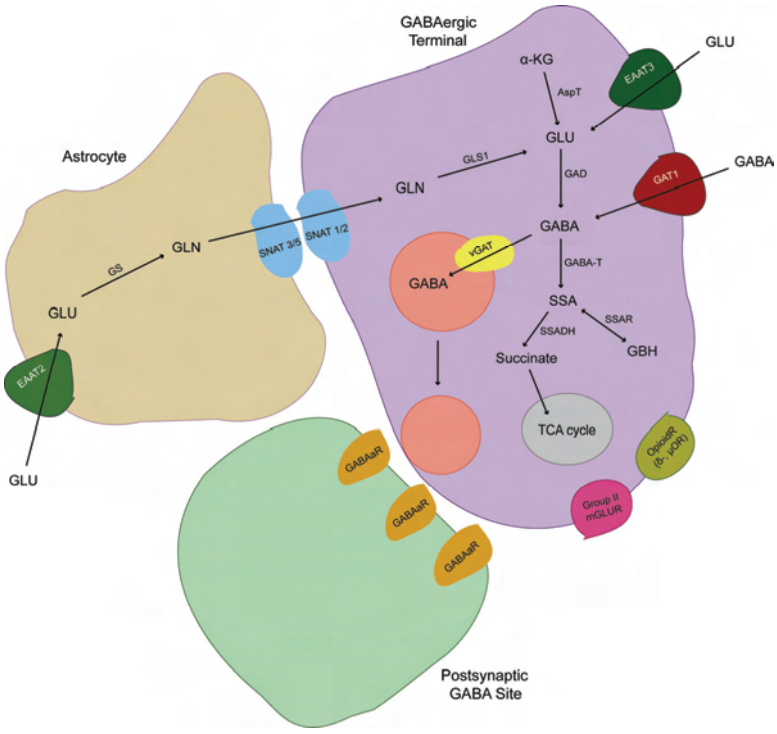

Figure 1. | **Metabolic pathways of GABA.** Acquisition of substrate for GABA synthesis, GABA packaging into synaptic vesicles, and breakdown of GABA by respective proteins.

compartments of excitatory neurons, and at axon terminals of GABAergic neurons[11, 12]. SNAT1 and 2 are members of the sodium coupled neutral amino acid transporter (SNAT) family and are expressed on excitatory and inhibitory neurons[13]. Because of their substrate affinity and the high ambient concentration of glutamine in the brain, SNAT1 and 2 are likely the major glutamine uptake pathway for neurons[14].

Astrocytes express glutamate transporters EAAT1 and 2 (also known as GLAST and GLT-1, respectively), of which EAAT2 is the major transporter for the clearance of synaptically released glutamate[15], as well as SNAT3 and 5[13]. A glutamine-glutamate-GABA cycle has been proposed[1] in which glutamate released from neurons is taken up by astrocytes, converted to glutamine with the enzyme glutamine synthetase and subsequently transported back to neurons (**Figure 1**). In neurons, glutaminase type I (GLS1) has been proposed to be the enzyme that converts glutamine to glutamate [1]. Studies have suggested that the majority of neurotransmitter glutamate is recycled by GLS1 with minimal contribution of *de novo* synthesis of glutamate from α-ketoglutarate in excitatory neurons[16, 17]. Interestingly, when GLS1 was knocked out, GABA levels were not reduced[18], suggesting alternative metabolic pathways for GABA synthesis must be present in inhibitory neurons.

In acute hippocampal slices, pharmacological inhibition of either EAATs or SNATs results in a rapid reduction of GABA vesicle content, as measured by changes in miniature inhibitory postsynaptic current (mIPSC) amplitudes[3, 19]. These results suggest that a dynamic equilibrium exists between GABA synthesis and vesicular filling, and consequently that inhibitory synaptic strength is directly regulated by two different substrate supply pathways. These results also suggest that substrate supply is a key regulatory point in the determination of inhibitory synaptic strength.

THERAPEUTIC IMPLICATIONS OF REGULATING INHIBITORY NEUROTRANSMISSION: WHAT KNOCKOUT STUDIES TELL US ABOUT THE ROLE OF KEY PROTEINS INVOLVED IN THE REGULATION OF GABA SYNTHESIS

(summarized in **Table 1**)

The importance of the regulatory points of GABA synthesis is revealed by knock-out mouse studies. After glutamate is cleared by glutamate transporters on glia, glutamine synthetase (GS) converts glutamate to glutamine to recycle neurotransmitter for both excitatory and inhibitory neurotransmission. In the context of an entire organism, the conversion of glutamate to glutamine results in detoxification of ammonia. As expected, knocking out GS has a severe phenotype, with death occurring at embryonic day 3.5[20]. Since GS has a global role beyond the central nervous system, it is not likely to be a good therapeutic target to alter GABA metabolism.

As mentioned above, GLS1 is the enzyme thought to convert glutamine to glutamate in neurons. The GLS1 knock-out mouse dies within a day of birth, is slightly smaller than wild type, has impaired respiratory function, and is deficient in goal-directed behavior[18]. It is thought that respiratory acidosis causes respiratory impairment and subsequent death.

There are 2 isoforms of GAD, GAD65 and GAD67, named after their molecular weights of 65 and 67 kDa, respectively. These 2 isoforms are encoded by independent genes and have different subcellular localizations in inhibitory neurons. As mentioned earlier, GAD65 is found at synapses. One study showed that GAD65 knock-out mice are more likely to develop seizures than wild type mice[21]. A different GAD65 knock-out mouse had an epileptic phenotype characterized by spontaneous seizures that led to death[22]. This mouse also showed increased anxiety-like behaviors and diminished response to anxiolytics[23], pre-pulse inhibition deficits[24], upregulation of the vesicular GABA transporter, and increased cytosolic GABA transport into synaptic vesicles[25].

GAD67 localizes to the cell soma of inhibitory neurons. The GAD67 knock-out mouse shows a

reduction in GABA levels throughout the brain, a reduction in GAD activity, and severe cleft palate, which leads to death within 24 hours of birth[26]. It is thought that the reduction of GABA levels in the GAD67 knock-out mouse brainstem to 30% of wild type leads to a malfunction in the respiratory control system and subsequent death[27]. The GAD65/GAD67 double knock-out mouse dies after birth due to cleft palate. GABA levels are low in this mouse[28] and another study with a different GAD65/GAD67 double knock-out mouse determined that GABA synthesis is absent[29].

The vesicular GABA transporter (vGAT) fills synaptic vesicles with both inhibitory neurotransmitters GABA and glycine. Study of a vGAT knock-out mouse showed that this mouse is incapable of executing vesicular release of GABA and glycine[29].

GAT1 is the predominant transporter responsible for GABA reuptake into inhibitory terminals, which allows for termination of GABA transmission. The GAT1 knock-out mouse showed reduced anxiety-like and depression-like behaviors[30], as well as decreased aggression[31], tremor, ataxia, nervousness, and increased extracellular GABA levels, which led to enhanced tonic inhibition and diminished phasic inhibition[9]. GAT1 has been targeted therapeutically by drugs, such as tiagabine, to treat epilepsy and anxiety[32].

GABA-T and SSADH perform a 2-step enzymatic breakdown of GABA. While there is no knock-out mouse for GABA-T, drugs that inhibit GABA-T, such as vigabatrin, have been used to increase GABA levels in the brain. Vigabatrin is not a drug of choice for epilepsy treatment because it often causes visual field defects[33]. The SSADH knock-out mouse exhibits ataxia, absence-like seizures with ictal behavior characterized by facial myoclonus, vibrissal twitching, and frozen immobility at 2 weeks. At this time, the absence seizures become more severe evolving into generalized convulsive seizures that progress into lethal status epilepticus[34, 35]. This mouse provides a good model for SSADH deficiency seen in humans[36], which is characterized by absence seizures and mental retardation.

THE ROLE OF EAAT3 ON INHIBITORY NEUROTRANSMISSION, SEIZURES, AND EPILEPSY

A role for EAAT3 as a critical regulator of neuronal excitability *in vivo* was demonstrated using antisense knock-down of the transporter[37-42]. In this study, antisense oligonucleotides to EAAT3 were infused into the lateral ventricles of rats, adjacent to the hippocampus. The animals developed spontaneous seizures corresponding to the time course of EAAT3 protein level reduction. Biochemical analysis confirmed reduced GABA content and impaired GABA synthesis in hippocampal tissue from treated animals. These results suggest that EAAT3 mediates an endogenous negative feedback mechanism whereby increased extracellular glutamate enhances GABA synthesis and inhibitory synaptic strength. The EAAT3 knock-out mouse did not have an epileptic phenotype, but it did develop dicarboxylic aminoaciduria and behavioral abnormalities[43]. In addition, this mouse develops glutathione deficiency and shows age-dependent

Table 1 | **Summary of knock-out studies of key regulatory points in GABA metabolism.**

Protein(s)	Cellular Localization	Function	KO Phenotype
Glutamic Acid Decarboxylase (GAD65)	Inhibitory neuron terminals	decarboxylates glutamate to synthesize GABA	increased seizure susceptible, epilepsy (spontaneous seizures leading to death), increased anxiety, LTD absence in visual cortex, PPI deficits, vGAT upregulation, increased cytosolic GABA transport into synaptic vesicles
Glutamic Acid Decarboxylase (GAD67)	Inhibitory neuron soma	decarboxylates glutamate to synthesize GABA	reductions in GABA levels throughout the brain, reduced GAD activities, cleft palate leading to death on P0, reduction of GABA levels in brainstem to 30% of wild type leading to abnormal respiratory patterns
GAD65/67	Inhibitory neurons	decarboxylates glutamate to synthesize GABA	die after birth due to cleft palate, GABA levels are scarce, absence of GABA synthesis
Vesicular GABA transporter (vGAT)	Inhibitory neuron terminals	transports GABA and glycine into synaptic vesicles	absence of GABA and glycine vesicular release
GABA transporter (GAT1)	Inhibitory neuron axons and terminals	GABA reuptake, terminate GABA transmission	reduced depression- and anxiety-like behaviors, decreased agression; tremor, ataxia, nervousness, increased GABA-induced tonic conductance in cerebellum, altered behavioral responses to alcohol, enhanced extracellular GABA levels, enhanced tonic, diminished phasic inhibition
GABA transaminase (GABA-T)	Inhibitory neuron terminals	breaks down GABA into succinic semialdehyde (SSA)	N/A
Succinic Semialdehyde Dehydrogenase (SSADH)	Inhibitory neuron terminals	terminates GABA catabolism by breaking down SSA into succinate	ataxia, absence-like seizures with ictal behavior characterized by facial myoclonus, vibrissal twitching, and frozen immobility at 2 weeks, absence seizures become more severe evolving into generalized convulsive seizures that progress into lethal status epilepticus
Glutamine Synthetase (GS)	Astrocytes	removes ammonia and glutamine (converts glutamate to glutamine)	dies at embryonic day 3.5
Glutaminase (GLS1)	Excitatory and inhibitory neurons	converts glutamine to glutamate	dies within 24 hours of birth, slightly smaller, impaired respiratory function, deficient in goal-directed behavior
SNAT3/5	Astrocytes	transports glutamine out of astrocytes	N/A
SNAT1/2	Neurons	takes up glutamine into neurons	N/A
Excitatory Amino Acid Transporter 3 (EAAT3)	Excitatory and inhibitory neurons	glutamate reuptake, provides substrate for GABA synthesis	decarboxylic aminoaciduria, behavioral abnormalities, glutathione deficiency, age-dependent neurodegeneration
Excitatory Amino Acid Transporter 2 (EAAT2)	Astrocytes	clears and recycles most of the extracellular glutamate	seizures, epilepsy

neurodegeneration[44]. The discrepancy in the phenotypes between the knock-out mouse and the knock-down rats, in particular the epileptic phenotype, has been attributed to a compensatory upregulation of a glutamate transporter homologous to EAAT3 in the knock-out mouse[4].

Several studies have investigated changes in EAAT3 expression in a variety of chronic epilepsy models and in human epileptic brain. Most studies of seizure models have looked at EAAT3 expression changes at least 24 hours after seizure induction[45], and these studies have examined changes at the tissue level which would predominantly reflect expression changes in the more numerous excitatory neurons. Moreover, the results of these studies were inconsistent, possibly due to differences in the epilepsy models, measurements (mRNA vs. protein) and regions examined. The only study of acute changes showed that EAAT3 protein in hippocampal pyramidal neurons appears to internalize 6 hours after kainic acid seizure induction[46]. No study has examined the expression of EAAT3 by inhibitory neurons in seizures and epilepsy. Because EAAT3 is expressed at postsynaptic sites on excitatory neurons and presynaptically on inhibitory neurons, it seems reasonable to hypothesize that seizure activity will have distinct effects on these two pools of transporters.

Recent evidence suggests that one function of glutamate transporters on inhibitory neurons, potentially EAAT3, may be the dynamic regulation of inhibition by extracellular glutamate levels[47]. Glutamate has been reported to increase extracellularly prior to seizure onset in human brain[48]. Therefore, EAAT3 may function to prevent the onset of seizures or to curtail seizure activity once started through enhancement of inhibition.

REGULATION OF EAAT3 SURFACE EXPRESSION AND GLUTAMATE UPTAKE BY SIGNALING CASCADE MOLECULES

Signaling cascade-mediated regulation of EAAT3 in inhibitory neurons could allow for modulation of inhibitory neurotransmission. A number of studies reported that EAAT3 activity is regulated by signaling cascade molecules. Most of these studies used heterologous settings including the C6 glioma cell line, which expresses EAAT3 endogenously. Regulation of neuronal EAAT3 in an endogenous setting and its effects on neuronal glutamate uptake are less well described. One study demonstrated functional upregulation of EAAT3 activity following induction of long term potentiation in the hippocampus and subsequent translocation of the protein from a cytosolic to a membrane compartment[49].

Glutamate uptake via EAAT3 is increased by non-specific activation of protein kinase C (PKC) with phorbol-12,13-myristate (PMA) in C6 glioma cells[50] and in primary neuronal cultures[46, 50]. This increase in glutamate uptake is associated with a rapid increase in EAAT3 surface expression in both C6 glioma cells and neurons[46]. Bisindolylmaleimide II (Bis II), a PKC inhibitor, completely blocks the PMA-induced glutamate uptake, but has no effect on basal glutamate uptake levels[51]. PKC activation mimicked the LTP induced upregulation of EAAT3[49].

PKC activity is mediated by a family of three subgroups (classic, novel, and atypical PKCs) each having unique properties. Classic PKC (cPKC) subtypes, which include three members (α, β, and γ), require calcium as a co-factor and are activated by diacylglycerol (DAG) and phorbol esters. In both C6 glioma cells and cortical neurons, the PMA-induced increase in EAAT3 activity was blocked with Gö6976 (10 μM), a selective inhibitor of cPKC subtypes[52]. Of the three cPKC subtypes, C6 glioma cells only express PKCα, suggesting that PKCα is the cPKC subtype that plays a role in the regulation of EAAT3 activity. In addition, C6 glioma cells treated with PMA showed a direct interaction between PKCα and EAAT3 on the cell surface[52]. Rat brain synaptosomes show basal EAAT3-PKCα association in the absence of PMA, while PMA treatment induced additional EAAT3-PKCα association. Both effects in synaptosomes are blocked by PKC antagonists suggesting the association may be triggered by endogenous stimulation of PKC activity under physiological conditions[53].

Phospatidylinositol 3-kinase (PI3K) has also been shown to regulate EAAT3 activity. Wortmannin, an irreversible PI3K inhibitor, decreases glutamate uptake and EAAT3 cell surface expression in C6 glioma cells within minutes. Platelet-derived growth factor (PDGF), which stimulates PI3K activity, increases both the activity and cell surface expression of EAAT3. The PDGF-mediated increase in EAAT3 activity is not blocked by the PKC antagonist BisII, and the PMA-mediated increase in glutamate uptake is not blocked by wortmannin suggesting that at least two independent signaling pathways regulate EAAT3 activity[50].

Protein kinase A (PKA) has been shown to regulate EAAT3 activity. In primary neuronal cultures glutamate uptake and EAAT3 surface expression decrease after treatment with H89, a PKA inhibitor. The H89-mediated decrease in glutamate uptake was counteracted by pre-treating cells with forskolin, a PKA activator[54].

As mentioned earlier, EAAT3 is expressed at GABAergic terminals and glutamatergic postsynaptic sites, but there are few studies that examined the functional regulation of EAAT3, and these studies have primarily looked at the postsynaptically

localized EAAT3. To our knowledge, no functional studies of EAAT3 at GABAergic terminals have been conducted.

REGULATION OF EAAT3 ACTIVITY BY PRESYNAPTIC RECEPTORS AT GABAERGIC TERMINALS: METABOTROPIC GLUTAMATE RECEPTORS AND OPIOID RECEPTORS

Metabotropic glutamate receptors (mGluRs) have multiple effects on interneurons through their actions on somata and axon terminals. In general, group I mGluRs (mGlu1 and mGlu5) are located on somatodendritic compartments, and group II/III mGluRs are located on presynaptic terminals, although there are many exceptions[55]. Presynaptic mGluRs on inhibitory terminals are activated by glutamate that is released from neighboring excitatory synapses[56]. When extracellular glutamate levels are sufficient to reach transporters on inhibitory terminals, it is likely that presynaptic mGluRs would be activated as well. mGlu1 agonists activate PKC in hippocampal pyramidal cells[57], but the signaling pathways activated in interneurons are not known. Interestingly, group I agonists are generally pro-convulsant *in vitro* and in animal models[58]. Investigation of possible regulation of EAAT3 activity on GABAergic neurons by mGluRs may provide important insights into the endogenous signaling mechanisms underlying a crosstalk between excitatory and inhibitory neurotransmission.

Opioid receptors are members of the superfamily of G-protein-coupled receptors that utilize inhibitory G-proteins ($G_{i/o}$). After $G_{i/o}$ are stimulated by opioid receptors, multiple effectors are activated including adenylyl cyclase and mitogen-activated protein kinase[54, 59]. Also, activation of $G_{i/o}$ leads to inhibition of cAMP production and PKA activity[60]. Inhibition of PKA has been associated with decreases in glutamate uptake and glutamate transporter surface expression levels by neurons[50]. Of the three well-characterized opioid receptors (Mu-, Delta-, and Kappa-opioid receptors), Mu-opioid receptor (MOR) and Delta-opioid receptor (DOR) are highly expressed in cortex[61, 62] and hippocampus[63-65]. While it seems that DOR is expressed by both excitatory and inhibitory neurons[66], MOR is preferentially expressed in axonal and somatodendritic compartments of GABAergic neurons in the hippocampus. In addition, both MOR and DOR localize to GABAergic neurons in dissociated cortical and hippocampal cultures[67].

Recently, it was shown in EAAT3-expressing *Xenopus* oocytes that co-expressing increasing amounts of DOR decreased glutamate uptake and EAAT3-mediated currents. In addition, DOR and EAAT3 can be co-immunoprecipitated and co-localized in both *Xenopus* oocytes and in rat cultured hippocampal neurons, suggesting a direct interaction between EAAT3 and DOR. Activation of DOR with pre-treatment of [D-Pen2,5]-enkephalin (DPDPE), a DOR agonist, counteracted the reduction in glutamate uptake and EAAT3-mediated current in *Xenopus* oocytes, and co-localization in both *Xenopus* oocytes and hippocampal neurons[68]. It is possible that DOR inactivates EAAT3 by its direct interaction and when DOR is stimulated by DPDPE, EAAT3 is released and allowed to increase its activity. This is further indication that EAAT3 could be regulated by G-protein coupled receptors. In addition, strong evidence suggests that protein kinase B (Akt), a downstream target of PI3K, regulates EAAT3 activity[69]. Akt is a downstream target of DOR in T cells making DOR a strong candidate for the regulation of EAAT3[70]. Whether MOR also regulates EAAT3 activity has not been investigated, however, its selective expression by GABAergic neurons suggests the possibility that, if it does, it may provide a signaling mechanism to selectively regulate EAAT3 on inhibitory neurons. Therefore, from a potential therapeutic perspective, MOR may be the most interesting candidate for the regulation of glutamate transporter activity in GABAergic neurons.

CONCLUSIONS

This review highlights the importance of the regulation of neurotransmitter GABA metabolism. In order to make progress towards the development of novel therapeutic targets for inhibitory neurotransmission, candidate therapeutic targets at GABAergic terminals, such as EAAT3, must be studied. Through EAAT3's possible regulation by signaling cascade molecules and specific receptors, the activity of EAAT3 could be manipulated in order to alter glutamate uptake, GABA synthesis, and ultimately, inhibitory neurotransmission. Additionally, regulatory points in GABA synthesis, such as GAD and vGAT, which can adjust GABA levels and alter inhibitory neurotransmission, need to be further explored.

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FURTHER INFORMATION

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