SVETLANA MOLCHANOVA

Release and Neuromodulatory Effects of Taurine in the Rodent Striatum

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Supervised by
Professor Pirjo Saransaari
University of Tampere
Emeritus Professor Simo S. Oja
University of Tampere

Reviewed by
Docent Irma Holopainen
University of Turku
Docent Tarja Stenberg
University of Helsinki

Distribution
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P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 3551 6055
Fax +358 3 3551 7685
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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following communications, referred to in the text by the Roman numerals I-V.

I. Molchanova S, Oja SS, Saransaari P (2004): Characteristics of basal taurine release in the rat striatum measured by microdialysis. Amino Acids 27: 261-268.

II. Molchanova SM, Oja SS, Saransaari P (2005): Mechanisms of enhanced taurine release under Ca^{2+} depletion. Neurochem Int 47: 343-349.

III. Molchanova S, Kööbi P, Oja SS, Saransaari P (2004): Interstitial concentrations of amino acids during global forebrain ischemia and potassium-evoked spreading depression. Neurochem Res 29: 1519-1527.

IV. Molchanova SM, Oja SS, Saransaari P (2006): Taurine attenuates D-[^{3}H]aspartate release evoked by depolarization in ischemic corticostriatal slices. Brain Res 1099: 64-72.

V. Molchanova SM, Oja SS, Saransaari P. (2006): Inhibitory effect of taurine on veratridine-evoked D-[^{3}H]aspartate release from murine corticostriatal slices: involvement of chloride channels and mitochondria. Brain Res, in press.

ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular concentration of Ca^{2+}
aCSF	artificial cerebrospinal fluid
AMPA	2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
DIDS	diisothiocyanostilbene-2,2'-disulfonate
EGTA	ethylene-bis(oxyethylenenitrile)tetraacetic acid
GABA	γ-aminobutyric acid
GES	guanidinoethanesulfonate
GluT	glutamate transporter
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
HPLC	high-performance liquid chromatography
KRH	Krebs-Ringer-HEPES buffer
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
OPA	*o*-phthaldialdehyde
SD	spreading depression
SITS	4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid
TAG	6-aminomethyl-3-methyl-4*H*-1,2,4-benzothiadiazine-1,1dioxide
TauT	taurine transporter
THBA	DL-threo-β-hydroxyaspartate
TTX	tetrodotoxin
VSCC	voltage-sensitive calcium channel
VSChC	volume-sensitive chloride channel
VSSC	voltage-sensitive sodium channel

ABSTRACT

Taurine is a sulfur-containing amino acid, considered to be an inhibitory neuromodulator in the mammalian brain. However, little is known regarding the regulation of extracellular concentrations of taurine and the molecular target(s) of its action. This study describes the properties of basal and evoked release of taurine, as well as its effect on the release of the excitatory neurotransmitter glutamate. The corticostriatal system of the rodent brain was chosen as the target of the study, mainly because it contains high amounts of taurine and powerful glutamatergic connections.

The basal and evoked release of taurine from the rat striatum was characterized using in vivo microdialysis. It was shown that the extracellular concentration of taurine in the striatum in vivo is 25.2 ± 5.1 µM. It is regulated by uptake by a taurine transporter and release through volume-sensitive chloride channels. Inhibition of synaptic vesicular release in the striatum in vivo by tetrodotoxin and omission of Ca^{2+} do not suppress, but enhance the release of taurine. Taurine release evoked by Ca^{2+}-free medium is activated by the altered function of voltage-sensitive Ca^{2+} channels and conducted by the reverse action of the taurine transporter. Neuronal depolarization and ischemia significantly elevate the extracellular concentration of taurine.

The inhibitory effects of taurine were studied in corticostriatal murine slices in vitro. It was shown that taurine suppresses the ischemic release of the non-metabolized glutamate analog D-[^{3}H]aspartate. Taurine affects the depolarization-evoked component of ischemic release but not hypo-osmotic and homoexchange-related components of release. The inhibitory effect of taurine on ischemia- and depolarization-evoked release is not blocked by competitive antagonists of γ-aminobutyric acid- and glycine-gated chloride channels. However, it is affected by the removal of Cl^- ions from the incubation medium and by blocking of the agonist-gated chloride channel pore. The effect of taurine was also significantly reduced by inhibition of mitochondrial Ca^{2+} uptake.

Based on the results obtained we conclude that the extracellular taurine pool is large, strictly regulated and affected by changes in neuronal activity. Taurine may inhibit the release of glutamate by activating membrane chloride channels and regulating Ca^{2+} concentration inside the cells. The findings show that although the properties of taurine release do not strictly conform to the criteria for neurotransmitters, it may serve a neuromodulatory function in the corticostriatal system of the rodent brain.

INTRODUCTION

Taurine (2-aminoethanesulfonic acid) is a non-essential amino acid abundant in the central nervous system (CNS) of mammals. Due to its numerous neuronal effects, taurine has been considered as a possible inhibitory neurotransmitter (Oja and Kontro, 1983; Oja and Saransaari, 1996). Indeed, many characteristics of taurine release, uptake and neuronal effects mimic those of neurotransmitters. Many specific questions, however, remain open, hampering definite conclusions as to the neurotransmitter or neuromodulatory functions of taurine. The most important of these questions are the regulation of the extracellular taurine concentration and the molecular mechanism underlying its neuronal effect.

In the case of classical neurotransmission, a transmitter should be released from neurons upon depolarization by Ca^{2+}-dependent exocytosis. The present data on the origin and mechanism of the evoked taurine release are inconsistent (Oja and Saransaari, 2000). However, to fulfil neuromodulatory functions in the brain, the extracellular taurine pool should be strictly regulated by neuronal activity regardless of the mechanisms involved. On the other hand, the target of taurine action in the brain has not been defined. Many of its neuronal effects are attenuated by γ-aminobutyric acid (GABA) and glycine receptor antagonists (Albrecht and Schousboe, 2005). However, it is not yet clear whether the inhibitory effects of taurine through GABA and glycine receptors are experimental artifacts due to the structural similarity of these amino acids and whether these phenomena take place in the brain of mammals under natural conditions. The possible presence of a specific taurine receptor in mammalian nervous tissue has also been considered, but such has not been purified and characterized.

The present study aimed to characterize taurine as a neurotransmitter or neuromodulator in the corticostriatal system of the rodent brain. The high concentration of taurine in the striatum and its heterogenic distribution allow one to assume taurine to serve neuromodulatory functions in this brain region. This thesis describes the properties of the basal and evoked release of taurine in the striatum, as well as its effect on the release of the excitatory neurotransmitter glutamate from the glutamatergic corticostriatal pathway. As taurine may protect neurons from glutamate-induced cell death under ischemic conditions, properties of its release and actions during brain ischemia were likewise characterized. The results obtained contribute to a better understanding of the function of taurine as a neuromodulator in the corticostriatal system of the rodent brain.

REVIEW OF THE LITERATURE

1. Content and distribution of taurine in the mammalian brain

Taurine is abundant in the mammalian CNS, with concentrations typically within the millimolar range (Palkovits et al., 1986; Huxtable, 1989; Puka et al., 1991). The content of taurine is comparable to that of GABA but lower than that of glutamate in the adult rodent brain (Huxtable, 1989). However, the relative concentration of taurine varies between species and brain structures. We have recently shown that in the striatum of anesthetized rats the taurine content is equal to that of glutamate, in contrast to the hippocampus, where there is significantly less taurine, and in both structures the concentration of GABA is significantly lower than that of taurine (Molchanova et al., 2006). The distribution of taurine in the brain is heterogeneous (Kontro et al., 1980; Palkovits et al., 1986; Pow et al., 2002). In the rat brain, high taurine levels are encountered in the cerebral cortex, striatum, cerebellum and supraoptic nucleus (Palkovits et al., 1986).

All cell types in the CNS appear to contain taurine (Huxtable, 1989). However, the localization of taurine differs in different brain areas and cell types. Elimination of neuronal cell bodies by kainic acid injection has significantly reduced the tissue content of taurine (Nicklas et al., 1979; Placheta et al., 1979). Injection of [^{3}H]taurine into the striatum leads to accumulation of the label in striatonigral, or medium-size densely spiny neurons, but not in neurons of other types (Clarke et al., 1983; Della Corte et al., 1990). In the substantia nigra, [^{3}H]taurine labels glial cells and neurites, but not neuronal cell bodies. In the supraoptic nucleus, immunostaining with antibodies raised against taurine conjugates has shown that taurine is mainly localized in glia and dendrites, but not in neurons (Decavel and Hatton, 1995). In the cerebellum, Purkinje cells contain taurine, in contrast to stellate and basket cell bodies (Ottersen et al., 1988).

In general, all this evidence on the marked heterogeneity of taurine distribution in the brain is not at variance with the hypothesis of its neurotransmitter function. Data on the heterogenic distribution of taurine transporter (TauT) and synthetic enzymes for taurine in the rat brain are also in accord with this assumption (Taber et al., 1986; Liu et al., 1992; Pow et al., 2002).

2. Synthesis and transport of taurine in nervous tissue

Mammalian species differ in their capability to synthesize taurine to meet the body's demands. In cats, taurine is an essential nutrient during the entire lifetime and especially during development (Sturman et al., 1985), while mice and rats are not dependent on the taurine supply in nutrition (Maar et al., 1995). Primates and man are able to synthesize taurine in adequate quantities in adulthood, but during the early phases of postnatal development taurine should be considered an essential nutrient for these species (Neuringer et al., 1985; Imaki et al., 1987).

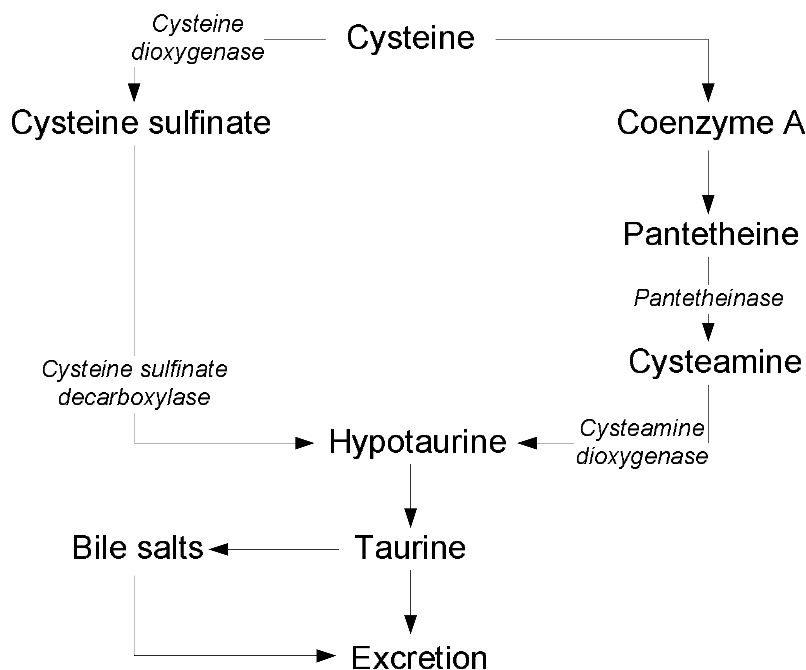


Figure 1. Metabolism of taurine in the mammalian tissues (Huxtable, 1986, 1992; Coloso et al., 2006).

The main pathways of taurine synthesis are shown in Figure 1. In animal tissues, the sulfur-containing amino acids methionine and cysteine serve as taurine precursors. Cysteine is either first oxidized by cysteine dioxygenase to 3-sulfinoalanine, which is then decarboxylated by sulfino decarboxylase (cysteinesulfinate decarboxylase) to yield hypotaurine (Huxtable, 1986) or transformed via coenzyme A to pantheteine and then to cysteamine, which is converted to hypotaurine by means of cysteamine dioxygenase (Coloso et al., 2006). The mechanism underlying the final step in taurine synthesis, oxidation of hypotaurine to taurine, has been claimed to occur only by means of non-enzymatic oxidation (Fellman and Roth, 1985), but the possibility of the involvement of enzyme(s) has also been considered (Oja and Kontro, 1981; Kontro and Oja, 1985). The brain thus contains the enzymatic machinery for taurine biosynthesis, but taurine levels in the brain depend mainly on the supply from other tissues, particularly from the liver (Beetsch and Olson, 1998; Stipanuk et al., 2002; Tappaz, 2004). Taurine is not broken down in the

mammalian organisms. The kidneys excrete taurine into the urine, the rate of excretion reflecting dietary intake and plasma levels (Chesney et al., 1985).

The pathway of taurine from blood to brain cells includes luminal uptake into endothelial cells, antiluminal release into the extracellular space and uptake into cells (Lallemand and De Witte, 2004). Taurine is a highly lipophilic compound and its exchange with the brain and blood is slow (Oja et al., 1976). It is transported to the brain by means of active uptake, as demonstrated in both cultured brain capillary endothelial cells and the whole brain in vivo (Bernabh et al., 1995; Tamai et al., 1995). The transport of taurine through the blood-brain barrier is saturable and Na^+- and Cl^--dependent. In vitro studies have shown that endothelial taurine uptake may be suppressed by elevated interstitial concentrations of taurine, as well as by other factors, including hypertonicity and oxidative stress (Kang et al., 2002; Kang, 2006). The taurine transporter mRNA and protein in the rat brain capillary endothelial cells have been detected and characterized (Kang et al., 2002).

The uptake of taurine into nerve cells is conducted by a similar mechanism. Taurine is transported into cells by an active uptake, which requires energy derived from the action of Na^+,K^+-ATPase and is dependent on Na^+ and Cl^- (Kontro and Oja, 1978; Oja and Kontro, 1983, 1984; Huxtable, 1989). Two or three Na^+ ions are required to transport one taurine molecule. Both neurons and glial cells accumulate taurine from the extracellular space. The uptake is saturable and consists of two components, low- and high-affinity. However, the numerical characteristics of taurine uptake vary between the subjects of study. The competitive inhibitors of taurine uptake are hypotaurine, β-alanine and guanidinoethanesulfonate (GES) (Lähdesmäki and Oja, 1973; Holopainen et al., 1983; Kontro and Oja, 1983; Barakat et al., 2002).

Taurine transporters have been cloned from the mouse and rat brain with K_m of 4.5 μM and 40 μM, respectively (Liu et al., 1992; Smith et al., 1992). Using the antisera generated to TauT, two of them have been demonstrated in the rat brain, confirming the existence of multiple types of TauT within one species (Pow et al., 2002). Both types of TauT share a high sequence homology to the glycine and GABA transporters with 12 transmembrane segments, indicating that they belong to the family of Na^+- and Cl^--dependent transporters (Liu et al., 1992; Smith et al., 1992; Tappaz, 2004). The transporters are differently distributed in the brain, with the highest expression of TauT in the striatum (Liu et al., 1992; Sergeeva et al., 2003). The activity of TauT is regulated by phosphorylation through the Ca^{2+}-dependent protein kinase A and the cyclic adenosine monophosphate (cAMP) -dependent protein kinase C, and the expression of the transporter protein is reduced by excessive amounts of taurine and enhanced by hypertonicity, tumor necrosis factor-α and nitric oxide (Tappaz, 2004).

It has been shown that neurotransmitter transporters may operate in the reverse mode, mediating the efflux of neurotransmitters into the extracellular

space (Bernath, 1992). The deregulation of the carrier may be evoked by changes in the Na^+ gradient across the plasma membrane. The outward current has been shown for the TauT expressed in Bergmann glia (Barakat et al., 2002). Na^+-free medium enhances taurine release via the reverse functioning of TauT in different brain preparations (Oja et al., 1985; Oja and Kontro, 1987; Takuma et al., 1996), including release under ischemic conditions (Saransaari and Oja, 1998, 1999a; Phillis and O'Regan, 2003). The *N*-methyl-D-aspartate (NMDA) - evoked release of taurine is also mediated by the reverse action of TauT (Saransaari and Oja, 1999b, 2003; Scheller et al., 2000b).

3. Taurine release from nervous tissue

The postulated neuromodulator or transmitter role of taurine in the brain (Oja and Kontro, 1983) implies that it is released through Ca^{2+}-dependent exocytosis. However, experimental studies have not unequivocally confirmed this assumption. Although taurine is enriched in the synaptic vesicle fractions (Kontro et al., 1980), no active uptake of taurine into synaptic vesicles has been found (Fykse and Fonnum, 1996). Taurine is released from nervous tissue in response to high concentrations of K^+ or other depolarizing agents (Solís et al., 1986; Kontro and Oja, 1987d; Holopainen et al., 1989; Semba et al., 1995; Estevez et al., 2000). This release has been shown to be partially Ca^{2+}-dependent, suggesting the involvement of the exocytotic pathway (Kontro, 1979; Oja et al., 1985; Kamisaki et al., 1993; García Dopico et al., 2004, Saransaari and Oja 2006). However, a number of other investigators have failed to show Ca^{2+}-dependency in the evoked taurine release (Holopainen et al., 1985; Solís et al., 1986; Rogers et al., 1991). The main factor hampering definitive conclusions on Ca^{2+} dependency is that Ca^{2+} depletion significantly enhanced the basal release of taurine in the studies in question. Nevertheless, when the K^+-stimulated release under Ca^{2+}-free conditions is compared to the basal release (Ca^{2+}-free conditions only), a significant reduction in the evoked release in the absence of Ca^{2+} is evident (Korpi and Oja, 1984; Oja et al., 1985; Oja and Kontro, 1987).

Taurine release may be mediated by the activation of glutamate receptors. Indeed, although glutamate and taurine are co-released by depolarizing stimuli, taurine release is slow in onset (Holopainen et al., 1989; Schousboe et al., 1990; Scheller et al., 2000a). Application of ionotropic and metabotropic glutamate receptor agonists evokes significant taurine release (Menéndez et al., 1993; Scheller et al., 2000a; Oja and Saransaari 2000; Saransaari and Oja, 2003). Endogenous glutamate release evoked by the inhibitors of the glutamate membrane carrier also amplifies the release of taurine, and glutamate receptor antagonists inhibit the release (Del Arco et al., 2000). Taurine release evoked by the glutamate receptor agonist NMDA is partly Ca^{2+}-dependent (Menéndez et al., 1993; Oja and Saransaari 2000). Nitric oxide (NO) synthase inhibitors also attenuate this release, suggesting the involvement of the NO/cyclic guanosine monophosphate (cGMP) pathway (Scheller et al., 2000b; Oja and Saransaari

2000; Saransaari and Oja, 2003). Such an assumption is supported by studies showing that cGMP and NO-generating compounds evoke the release of taurine from neuronal preparations while inhibitors of guanylate cyclase prevent this effect (Saransaari and Oja, 1999b, 2002; Scheller et al., 2000b; Watts et al., 2004). TauT inhibitors reduce the effects of NO-generating compounds, suggesting that taurine release evoked by activation of the NO/cGMP pathway is mediated by the reverse action of TauT (Chen et al., 1996; Saransaari and Oja, 1999b). However, NO donors reduce the depolarization-evoked release of taurine (Saransaari and Oja, 1999b). While several inhibitors of NO synthase reduce the NMDA-evoked release of taurine, their effects on the basal release of this amino acid are the opposite (Watts et al., 2004). Based on these data, it is possible to suggest that a NO-dependent pathway regulates taurine release, but the mechanisms of this regulation are very complicated. The difference in the effects of NO donors and NO synthase inhibitors may be related to different regulation mechanisms in neurons and glial cells.

Various pathological conditions such as ischemia evoke taurine release through several release pathways (Oja and Saransaari, 2000). Many pathological conditions, including ischemia, are accompanied by brain edema. When cells swell in a hypo-osmotic environment, one means to restore the initial cell volume is to extrude osmotically active substances. Of the osmolytes, taurine is the most sensitive to osmotic perturbation (Schaffer et al., 2000; Pasantes-Morales et al., 2002), and application of exogenous taurine has been shown to enhance cell volume regulation (Kreisman and Olson, 2003). Under hypo-osmotic conditions taurine is released into the extracellular space (Oja and Saransaari, 1996; Hussy et al., 2000; Pasantes-Morales et al., 2002; Kreisman and Olson, 2003). This release is mediated by volume-sensitive chloride channels (VSChCs) (Pasantes-Morales et al., 1990; Estevez et al., 1999). Inhibitors of these channels have been shown to reduce taurine release under ischemic conditions (Phillis et al., 1997; Saransaari and Oja 1998; Phillis and O'Regan, 2003). However, the hypo-osmotic release pathway may also take part in the taurine release mediated by high K^+ depolarization (Saransaari, Oja, 1998). It has been shown that omission of Na^+ from the perfusion medium reduces the ischemia-evoked release of taurine from hippocampal slices, indicating the participation of the TauT-mediated pathway (Saransaari and Oja, 1998). Synaptic vesicular release may also be involved, since the inhibitor of voltage-sensitive sodium channels (VSSCs) tetrodotoxin (TTX) has reduced the release of taurine from striatal slices (Büyükuysal, 2004). Taurine release under ischemic conditions may be induced by membrane disruption due to the activation of phospholipases (Phillis and O'Regan, 2003). Taurine is also released by other pathological conditions such as oxidative stress (Saransaari and Oja, 2004). This release is likewise mediated by the reverse action of TauT and leakage through membrane chloride channels.

To conclude, taurine is released from nervous tissue in response to various stimuli, including membrane depolarization, hypo-osmotic and pathological

conditions. This release may be evoked by activation of glutamate receptors and the NO/cGMP pathway and mediated by VSChCs or TauT operating in the reverse mode. However, the exact mechanisms of taurine release during synaptic transmission are not yet known.

4. Neuromodulatory effects of taurine

Taurine exerts multiple effects in the neuronal tissues, including modulation of neuronal activity, osmoregulation, membrane stabilization, regulation of cellular calcium fluxes, influence on protein phosphorylation, neutralization of reactive oxygen species and modulation of membrane channels (Lombardini 1994; Shaffer et al., 2000; Foos and Wu, 2002; Schuller-Levis and Park, 2003; El Idrissi and Trenkner, 2004; Albrecht and Schousboe, 2005). The particular role of taurine has been shown during development (Sturman, 1993). However, despite extensive studies during recent decades, the cellular mechanisms underlying the effects of taurine remain unclear. The neuromodulatory function of taurine is considered to be mainly related to its ability to alter the neuronal membrane potential, to modulate neurotransmitter release and to regulate the concentration of Ca^{2+} inside the cells (El Idrissi and Trenkner, 2004). Since the aim of this thesis is related to the neuromodulatory properties of taurine, this function has received the main attention in this review.

Application of taurine enhances the influx of labeled Cl^- into incubated slices from different brain regions (Oja et al., 1990) and cerebellar granule cells in culture (El Idrissi and Trenkner, 2004), and increases chloride conductance as measured by whole-cell patch-clamp recordings in slices from the olfactory bulb (Belluzzi et al., 2004). Taurine attenuates the membrane depolarization evoked by application of glutamate (Wu et al., 2005). Upon acute application taurine depresses the evoked field responses in incubated corticostriatal slices (Chepkova et al., 2002). However, withdrawal of taurine evokes a long-lasting enhancement of corticostriatal and hippocampal synaptic transmission through a complex neuromodulatory mechanism (del Olmo et al., 2000b,c; Chepkova et al., 2002, 2005).

Taurine modulates the release of several neurotransmitters. It has been shown to modify the high K^+-induced release of amino acid transmitters, including glutamate, from nerve terminals isolated from the cerebral cortex (Kamisaki et al., 1993; Zheng, 2001). At variance with this, a microdialysis study on the substantia nigra has shown amplification of glutamate release due to taurine administration (García Dopico et al., 2004). Local perfusion of the taurine uptake blocker GES to the nucleus accumbens has led to an elevation in extracellular taurine levels and to a significant reduction in extracellular levels of aspartate, glutamate and glycine (Olive et al., 2000). Chronic modulation of the taurine content in the brain affects both inhibitory and excitatory neurotransmitter systems. Taurine-transporter gene knockout animals show higher $GABA_A$ receptor densities in the hippocampus and cerebellum and kainate receptor densities in the striatum, hippocampus and cerebellum

(Oermann et al., 2005). Chronic application of taurine increases susceptibility to kainate-induced seizures, since taurine-fed mice have elevated brain levels of glutamate and GABA, decreased expression of $GABA_A$ receptor subunits and increased expression of the GABA-synthesizing enzyme glutamate decarboxylase (El Idrissi, 2006). Chronic application of taurine continuously interacts with GABA receptors, leading to a compensatory decrease in their expression and a compensatory increase in GABA synthesis.

Many effects of taurine are mediated by the regulation of Ca^{2+} fluxes and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). When toxic concentrations of glutamate are applied, taurine enhances cell survival by lowering the $[Ca^{2+}]_i$ (El Idrissi and Trenkner, 1999, 2004; Zhao et al., 1999; Chen et al., 2001; Foos and Wu, 2002; Wu et al., 2005). Taurine has been shown to reduce the glutamate-evoked Ca^{2+} influx into cells (Chen et al., 2001; Wu et al., 2005) and Ca^{2+} release from the intracellular storage pools (Foos and Wu, 2002). The latter phenomenon may be connected to the regulation of Ca^{2+} sequestration by mitochondria, which constitute one of the main calcium intracellular storage pools. Indeed, taurine has been shown to affect the Ca^{2+} concentration in isolated brain and liver mitochondria and cultured cerebellar granule cells (Li and Lombardini, 1991; Palmi et al., 1999; El Idrissi and Trenkner, 2003). However, activation of putative taurine receptors has also been thought to take part in the molecular reaction chain leading to intracellular Ca^{2+} regulation by taurine (Foos and Wu, 2002; Wu et al., 2005).

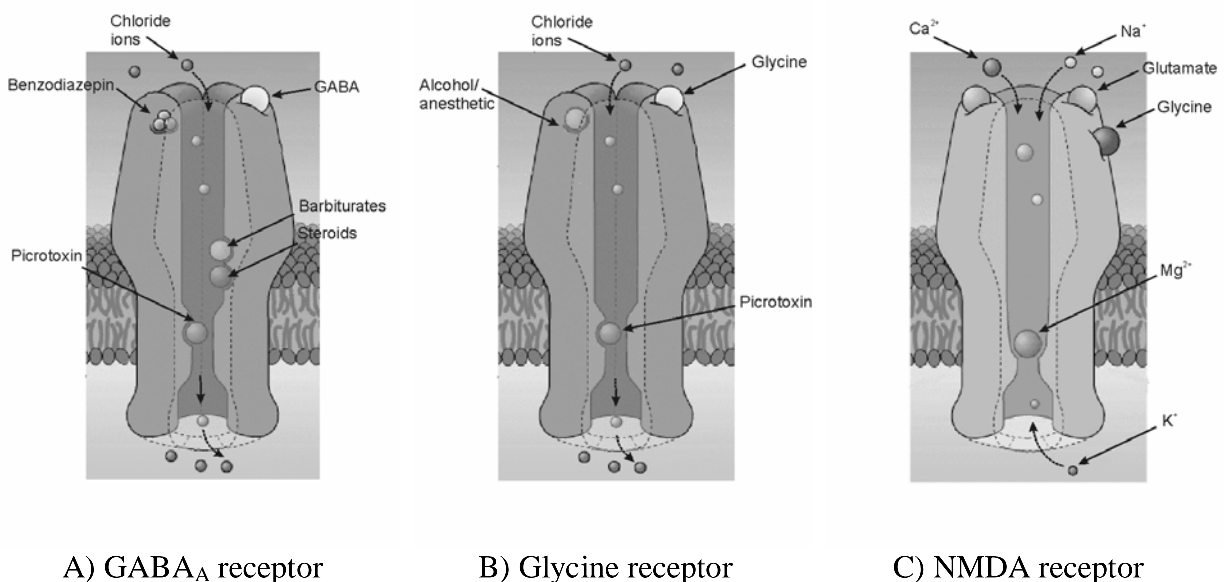
5. Interaction of taurine with membrane receptors

Taurine may serve as a partial agonist for $GABA_A$ and glycine receptors, illustrated in Figure 2 (A,B). Taurine-evoked chloride fluxes in brain slices, cerebellar granule cells and olfactory bulb slices are inhibited by the antagonist of $GABA_A$ receptors bicuculline (Oja et al., 1990; Belluzzi et al., 2004; El Idrissi and Trenkner, 2004). Taurine activates $GABA_A$ receptors in the hippocampal CA1 area and neocortex of the rodent brain, as well as receptors expressed in Xenopus oocytes injected with mouse brain messenger RNA (Horikoshi et al., 1988; del Olmo et al., 2000a; Yoshida et al., 2004). Taurine displaces binding of labeled GABA to the GABA-benzodiazepine receptor complex and inhibits [^{3}H]muscimol binding to purified $GABA_A$ receptors, suggesting that it acts on a GABA-binding site (Malminen and Kontro, 1986; Bureau and Olsen, 1991). However, the affinity of these receptors to taurine is less marked (del Olmo et al., 2000a, Barakat et al., 2002).

Glycine is an inhibitory neurotransmitter in the lower brain regions, even though glycine receptors have also been demonstrated in the striatum, hippocampus, substantia nigra and supraoptic nucleus (Ye at al., 1997; Darstein et al., 2000; Sergeeva and Haas, 2001; Chattipakorn and McMahon, 2002; Deleuze et al., 2005). These receptors are also activated by taurine, albeit with less potency compared to glycine. The effect of taurine on the glycine receptors is effectively antagonized by the competitive inhibitor strychnine and the

channel blocker picrotoxin, suggesting that taurine acts at the agonist-binding site (Jiang et al., 2004).

Various neuronal effects of taurine in the supraoptic nucleus are mediated by glycine receptors, which has prompted debate as to whether taurine is the major natural agonist of these receptors in this brain region (Hussy et al., 1997, 2000; Song and Hatton, 2003). The electrophysiological and immunocytochemical studies in the rat supraoptic nucleus show the absence of glycinergic transmission in this brain region, and the extrasynaptic localization of the glycine receptor clusters, which are located predominantly near the astroglial processes enriched with taurine (Deleuze et al., 2005). The nonsynaptically released taurine is suggested to be the main endogenous ligand for the glycine receptors in the immature neocortex (Flint et al., 1998). Taurine has also been shown to activate the glycine receptors in the ventral tegmental area of young rats (Wang et al., 2005). These receptors are activated by the tonically released ligand, which allows to suggest nonsynaptic nature of the released taurine/glycine. Electrophysiological studies of the strychnine-sensitive chloride currents in hippocampal organotypic slice cultures have shown that glycine receptors are expressed in certain cell types, but not activated during synaptic stimulation (Mori et al., 2002). Inhibition of TauT evokes activation of these receptors, whereas modulation of the extracellular glycine concentration is without effect. This evidence also suggests primary role for endogenous taurine in the activation of hippocampal glycine receptors.



A) $GABA_A$ receptor B) Glycine receptor C) NMDA receptor

Figure 2. Schematic illustration of some amino acid ionotropic receptors (Neuroscience, 2nd edition, edited by Purves et al.).

$GABA_A$ and glycine receptors belong to a superfamily of nicotinicoid receptors (Jentsch et al., 2002; Korpi et al., 2002; Cascio 2004; Lynch 2004). The receptors of this group are homologous ionotropic membrane channels mediating fast synaptic transmission in the CNS. In response to neurotransmitter binding they transiently open the selective pore, allowing passive movement of small ions down their electrochemical gradient (Figure 2A,B). Both $GABA_A$ and

glycine receptors are chloride membrane channels. These receptors are typically heteropentameric oligomers bearing a conservative disulfide loop in their extracellular ligand-binding domain (Cys-loop). $GABA_A$ receptors are composed of five subunits which belong to eight different classes, and the glycine receptor may include subunits belonging to five different classes (Jentsch et al., 2002; Korpi et al., 2002; Cascio 2004; Lynch 2004).

The possible presence of a specific taurine receptor in the nervous tissue of mammals has also been discussed. 6-Aminomethyl-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (TAG) has been found to selectively inhibit various physiological and biochemical effects of taurine, without significant effects on GABAergic or glycinergic systems (Martin et al., 1981; Girard et al., 1982; Okamoto et al., 1983; Wessberg et al., 1983; Kubo et al., 1993; Engelmann et al., 2001). On the other hand, TAG interferes with the effects of GABA agonists on the amphibian cortical cell membrane polarization and with the taurine uptake system in rat brain membranes, indicating the relative nonspecificity of its action (Yarbrough et al., 1981; Lewin et al., 1994). A putative specific taurine recognition site in synaptic membranes has been described (Kontro and Oja, 1987b; Wu et al., 1992, 2001; Frosini et al., 2003a,b). Sodium-independent binding of taurine to the mouse brain membranes is inhibited not only by the specific taurine antagonist TAG but also by glycine and GABA antagonists (Kontro and Oja, 1987). These findings are contradictory to those reported in another study, where all common agonists or antagonists of major amino acid neurotransmitter receptors, including strychnine, bicuculline and picrotoxin, were shown to have little effect on taurine receptor binding to isolated nerve endings (Wu et al., 2001). In the frog spinal cord, two taurine receptor subtypes have been surmised to exist on the basis of their pharmacological properties, differing from the responses to GABA and glycine (Kudo et al., 1988). Two proteins displaying the specific binding of taurine have been isolated from the olfactory organ of the lobster (Sung et al., 1996). Using rabbit brain membranes, four taurine analogs, including TAG, have been shown to bind specifically to the taurine binding site but not to $GABA_A$ or $GABA_B$ receptors (Frosini et al., 2003a). Three of these substances antagonize taurine effects on body temperature (Frosini et al., 2003b). All these findings bespeak the existence of a specific taurine receptor.

6. Neuroprotective effects of taurine

Taurine may serve as a neuroprotector in ischemic cell injury evoked by excessive extracellular glutamate concentrations. Exogenously applied taurine alleviates neuronal damage evoked by a variety of pathological conditions, including ischemia, epileptic seizures, energy metabolism perturbation and oxidative stress (O’Byrne and Tipton, 2000; Rivas-Arancibia et al., 2001; El-Abhar and Abd El Gawad, 2003; El Idrissi et al., 2003; Kingston et al., 2004; Wang et al., 2005). Taurine also protects neurons from glutamate-induced cell

death (Zhao et al., 1999; El Idrissi and Trenkner, 1999; Chen et al., 2001; Foos and Wu, 2002; Louzada et al., 2004; Wu et al., 2005).

Ischemic injury to nervous tissue involves a number of processes leading to cell death in vulnerable regions of the brain (Lipton, 1999). Energy failure during ischemia induces a massive release of the excitatory neurotransmitter L-glutamate (Benveniste et al., 1984; Globus et al., 1991, Phillis and O'Regan, 2003), which is considered to be the key factor in ischemic cell death. Glutamate is the main excitatory neurotransmitter in the mammalian CNS, implicated in one third of all excitatory connections (Watkins and Evans, 1981; Doble, 1999). It activates ionotropic and metabotropic membrane receptors (Ozawa et al., 1998; Doble, 1999). Ionotropic receptors are agonist-gated ion channels which belong to three types according to their agonist specificity: NMDA, 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate receptors. The NMDA receptors are permeable to calcium, sodium and potassium ions (Figure 2C). The AMPA and kainate receptors are mainly permeable to sodium and potassium ions, but some of their subunit compositions allow the calcium flow. The metabotropic receptors also consist of three types, which modulate the cellular metabolism via G-proteins and secondary messengers.

Glutamate and the agonists of glutamate receptors at high concentrations are toxic to nervous cells (Doble, 1999). Glutamate-evoked excitotoxicity takes part in the pathogenesis of many neurodegenerative diseases including ischemia, traumatic brain injury and Alzheimer's, Huntington's and Parkinson's diseases. The toxic effect of glutamate mainly stems from the activation of NMDA receptors, but other excitatory receptors may also be involved (Arundine and Tymianski, 2004). Overactivation of NMDA receptors initiates an increase in intracellular Ca^{2+} by means of both enhanced flow through the receptor pore and release from intracellular stores (Randall and Thayer, 1992). A persistent substantial elevation of $[Ca^{2+}]_i$ activates Ca^{2+}-dependent proteases, phospholipases and endonucleases and evokes excessive formation of reactive oxygen species, hence causing damage to macromolecules and cell death (Choi and Rothman, 1990, Arundine and Tymianski, 2004).

Taurine has been shown to attenuate pathological reactions induced by ischemia/reperfusion or toxic concentrations of glutamate. The neuroprotective effects of taurine may be mediated by its ability to hyperpolarize neuronal membranes, attenuate cell swelling, regulate calcium storage in cells and neutralize hypochlorous acid formed during oxidative stress (Oja and Saransaari, 1996; Schaffer et al., 2000; El Idrissi and Trenkner, 2004; Foos and Wu, 2002; Schuller-Levis and Park, 2003; Wu et al., 2005). Indeed, taurine attenuates the membrane depolarization evoked by the toxic concentrations of glutamate (Wu et al., 2005). The ability of taurine to rescue neurons incubated with glutamate is antagonized by inhibitors of both $GABA_A$ and glycine receptors (O'Byrne and Tipton, 2000; Louzada et al., 2004). Taurine prevents the increase in $[Ca^{2+}]_i$ mediated by glutamate by inhibiting both the influx of Ca^{2+} from outside the cell through calcium membrane channels and the release of Ca^{2+} from the internal

pools (Zhao et al., 1999; Foos and Wu, 2002; Wu et al., 2005). The generation of the reactive oxygen species upon glutamate excitotoxicity is suppressed by application of taurine (Boldyrev et al., 1999; Hilgier et al., 2003; Schuller-Levis and Park, 2003).

As excessive glutamate release is the major factor in the process of ischemic cell death, the ability of taurine to antagonize it is an essential issue in studies of the neuroprotective properties of taurine. The co-release of taurine and glutamate and the release of taurine evoked by the activation of glutamate receptors may be neuroprotective by balancing glutamate activity under cell-damaging conditions (Saransaari and Oja 1997, 1999a, Sheller et al., 2000a). To date there is a lack of evidence regarding the suppressive effect of taurine on pathological glutamate release. It has been shown that taurine blocks increased glutamate release during ethanol withdrawal in the nucleus accumbens of ethanol-dependent rats (Dahchour and De Witte, 2000). However, the ability of taurine to reduce the release of glutamate evoked by depolarizing agents (Kamisaki et al., 1993; Zheng, 2001) bespeaks a possible protective effect of taurine under pathological conditions.

AIMS OF THE STUDY

The aim of this study was to characterize taurine as a neuromodulator in the corticostriatal system of the rodent brain. The tasks involved in achieving this objective were:

1) to estimate the size of the extracellular taurine pool in the rat striatum in vivo and its regulation by volume-sensitive chloride channels and the taurine uptake system under resting conditions;
2) to describe the effect of decreased extracellular Ca^{2+} concentrations and the inhibitor of voltage-gated sodium channels on the extracellular concentration of taurine in the striatum of rats in vivo and to study the mechanisms of taurine release stimulated by Ca^{2+} omission;
3) to characterize the release of taurine from the rat striatum in vivo upon application of physiological (spreading depression, SD) and pathological (ischemia) stimuli;
4) to establish whether taurine has an inhibitory effect on glutamate release from incubated murine corticostriatal slices evoked by ischemia and activation of synaptic, hypo-osmotic and transporter-mediated release pathways using the non-metabolized glutamate analog D-[^{3}H]aspartate;
5) to characterize the involvement of agonist-gated chloride channels and Ca^{2+} regulation by mitochondria in the possible inhibitory effect of taurine.

MATERIALS AND METHODS

1. Experimental animals

The studies where the microdialysis technique was applied were carried out on male adult Sprague-Dawley rats weighing 250-300 g. The animals were anesthetized with halothane and decapitated at the end of experiments. Superfused corticostriatal brain slices were obtained from adult NMRI mice of both sexes. The mice were killed by decapitation and the brains were dissected out on ice. All animals were from Orion, Espoo, Finland, and were given food and water ad libitum and maintained in temperature-controlled rooms (22±1°C) with a constant relative humidity (50%) under a 12h light/dark cycle. The experimental procedures were in accordance with the European Community Directive for the ethical use of experimental animals. All efforts were made to minimize both the suffering and the number of animals used.

2. Chemicals

Taurine was purchased from Fluka (Buchs, Switzerland) and *o*-phthaldialdehyde (OPA), D-aspartate, DL-threo-β-hydroxyaspartate (THBA), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), tetrodotoxin (TTX), diisothiocyanostilbene-2,2'-disulfonate (DIDS), veratridine, ethylene-bis(oxyethylenenitrile)tetraacetic acid (EGTA), strychnine, bicuculline, GES, picrotoxin, rotenone, oligomycin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and amino acids used in standard solutions during high-performance liquid chromatography (HPLC) were from Sigma (St. Louis, MO, USA). D-[^{3}H]Aspartate (specific activity 1.48 PBq/mol) was purchased from Amersham International (Bristol, UK). The liquid scintillation cocktail OptiPhase "SuperMix" was produced by Wallac Oy (Turku, Finland). Other chemicals (including salts used for the preparation of artificial cerebrospinal fluid (aCSF), Krebs-Ringer-HEPES buffer (KRH) and phosphate buffer and reagents for HPLC were from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) and J.T. Baker (Deventer, Holland).

During microdialysis all substances were diluted in aCSF and applied through the microdialysis probe. In the course of efflux experiments all stock drug solutions were prepared in KRH, except for bicuculline, rotenone, CCCP and oligomycin, which were initially dissolved in dimethylsulfoxide (the final concentration of the solvent in the incubation medium did not exceed 0.01 %).

Table 1. The molecular targets of the chemicals used in the study

Compound	Function
Bicuculline	antagonist at $GABA_A$ receptor
GES	competitive inhibitor of TauT
Oligomycin	Inhibitor of mitochondrial ATPase
Picrotoxin	$GABA_A$ and glycine receptor channel blocker
Rotenone	Inhibitor of mitochondrial complex I
CCCP	Mitochondrial protonophore
SITS, DIDS	Inhibitors of VSChCs
Strychnine	antagonist at glycine receptor
THBA	competitive inhibitor of glutamate transporter (GluT)
TTX	Inhibitor of VSSCs
Veratridine	Activator of VSSCs

3. Brain microdialysis in vivo

Implantation of microdialysis probes took place on the day of experiments under halothane anesthesia. Rats were first anesthetized with 4% halothane in air and placed in a stereotaxic frame (David Kopf Instruments, Düsseldorf, Germany). The concentration of halothane was then reduced to 0.8-1.2% and body temperature kept at 37.5°C with a heating pad. Microdialysis probes of concentric design (4 mm length, 0.5 mm outer diameter) were implanted in the striatum. In most experiments two probes were implanted in the right and left striatum. The stereotaxic coordinates were as follows: +0.5 (anterioposterior axis); ±3 (mediolateral axis); -6.5 (dorsoventral axis); relative to the bregma and dural surface, according to the atlas of Paxinos and Watson (1996). In the experiments which included global cerebral ischemia only one probe was used. It was fixed to the skull with screws and dental cement. In other cases, the stereotaxic holders kept the probes in place during the experiments.

The probes were perfused with a CMA-102 pump (CMA/Microdialysis AB, Stockholm, Sweden) at a rate of 2 µl/min with aCSF containing (mM): 120 NaCl, 2.8 KCl, 25 $NaHCO_3$, 1.2 $CaCl_2$, and 1.0 $MgCl_2$, at pH 7.2, adjusted by bubbling of the solution with 5% CO_2. Sampling commenced at least 1.5 hour after probe implantation. Thereafter, samples of the dialysate were collected every 20 min and stored at -20°C until analyzed.

4. Animal model of global forebrain ischemia

All procedures were conducted after microdialysis probe implantation under halothane anesthesia. The carotid arteries were exposed and a catheter (PE 50, Clay-Adams, Parsippany, NJ, USA) inserted into the right femoral artery in order to measure the mean arterial blood pressure and for withdrawal of blood.

Forebrain ischemia was induced by bilateral carotid occlusion with controlled hypotension. The mean arterial blood pressure (mean basal value 96.3±2.1 mmHg, III) was halved by withdrawal of blood into a heparinized syringe (mean value in ischemic conditions 55.7±1.8 mmHg, III). Immediately after establishing hypotension both carotid arteries were clamped with clips. After a 20-min ischemic period the clips were removed and mean arterial blood pressure increased close to basal level.

5. High-performance liquid chromatography of amino acids

The amino acids were assayed in dialysates kept frozen and thawed immediately prior to the analyses. The concentrations of aspartate, glutamate, asparagine, serine, glutamine, glycine, threonine, alanine, taurine and GABA were measured by HPLC with fluorescent detection after precolumn derivatization with OPA using the system of Shimadzu Scientific Instruments (Kyoto, Japan). Derivatization was performed in a SIL-10AD autoinjector with the OPA reagent (OPA, 0.4 g/l, 0.25% mercaptopropionic acid and 2% methanol in 0.29 M borate acid, pH 10.4). The OPA reagent (26 µl) was added to the sample (56 µl), mixed, incubated for 2 min at 4°C, and then injected into the column. The amino acid derivatives were separated using a C18-HC column, ODS 2.5 µm packing, 4.6x250 mm (Waters, Elstree, Hertfordshire, UK) equipped with a precolumn (4x6 mm). The flow rate was set at 0.8 ml per min and the low-pressure gradient mode used for elution. The mobile phases were 10% methanol and 2% tetrahydrofuran in 0.067 M phosphate buffer, pH 6.8 (A), and 60% methanol and 2% tetrahydrofuran in water (B). The concentration of B during separation was gradually increased from 3% to 100% and then dropped to zero. Fluorescence was measured with an RF-10A detector using excitation and emission wavelengths set at 340 nm and 450 nm, respectively. The concentrations of amino acids in dialysate were calculated using external and internal standards with VPclass5 software. The detection limit for all amino acids measured was at least five times lower than the concentration of the lowest standard used (0.2 µM).

6. Efflux of D-[^{3}H]aspartate from superfused brain slices

Murine brain slices 0.4 mm thick, containing the sensorimotor cortex and striatum, were prepared with a McIlwain tissue chopper on a cold plate. The slices were then transferred into preoxygenated KRH solution containing (mM) 126 NaCl, 1.3 $MgSO_4$, 1.3 NaH_2PO_4, 5.0 KCl, 0.8 $CaCl_2$, 15 *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES) and 10 D-glucose (pH 7.4 adjusted with NaOH, calculated osmolarity 334 mOsm), and loaded with D-[^{3}H]aspartate (75 µM**,** 111 MBq/l, in the presence of 0.01 mM unlabeled D-aspartate). The preloading was performed during 30 min at 37°C under O_2 atmosphere. Thereafter, the excessive label was washed out with warm oxygenated KRH and the slices placed in 1 ml chambers. The slices were superfused with KRH (eight chambers in parallel) delivered at a rate of 0.25

ml/min at 37°C under O_2 atmosphere. The first fraction of superfusate was collected during 20 min and this experimental period was regarded as the recovery period. Thereafter, fractions were collected every 2 min. At the end of superfusion, the slices were homogenized in 5% trichloracetic acid and centrifuged. Aliquots of the supernatant and superfusate fractions were mixed with the liquid scintillation cocktail and subjected to radioactivity measurement in a liquid scintillation counter, 1450 Microbeta PLUS (Wallac Oy, Turku, Finland). The counts were corrected for quenching with the aid of external standardization, using Wallac 1450 MicroBeta 4.0 software.

7. Experimental set-up, data analysis and statistics

In vivo microdialysis was used to study the basal and evoked release of taurine in the striatum of rats. To ascertain the actual concentration of taurine in the extracellular space the zero net flux approach was applied (Lönnroth et al., 1987). At the beginning of the experiments three 20-min basal samples were collected. Thereafter, the probes were perfused with aCSF containing randomly 1, 5, 10, 30 and 50 µM taurine. Three samples were collected from each perfusate solution. Administration of the next solution commenced 20 min after the previous one. The basal level of taurine in the dialysate was calculated as an average ± S.E.M. from the first three samples collected in each experiment after perfusion with aCSF. The actual concentration and extraction fraction of taurine were then obtained using linear regression analysis from the data at different taurine concentrations in the perfusion fluid (Harris, 2003).

In the course of the study of the characteristics of basal taurine release, three basal samples of dialysate were collected at the beginning of the experiment. Thereafter, the modified aCSF or aCSF containing various inhibitors was perfused via the microdialysis probes from the fourth sample onwards. The mean concentration of amino acids in the first three samples was taken as 100%. The changes in amino acid concentrations evoked by treatments were then calculated as percentages of this baseline value. The data obtained were compared with the corresponding control values from separate experiments. The statistical significance of differences was estimated using Student's *t*-test. The level of significance was set at 0.05.

Taurine release evoked by global forebrain ischemia or SD was also studied by in vivo microdialysis. After three basal samples ischemic or depolarization conditions were evoked for 20 or 40 minutes, respectively. Thereafter, normal conditions were reestablished. The statistical significance of differences was estimated as previously described, comparing the data with basal and control taurine concentrations.

Superfused murine corticostriatal slices, preloaded with D-[^{3}H]aspartate, were used in a study of the effect of taurine on the evoked glutamate release. The first five samples of the incubation medium were collected for estimation of the basal efflux rate of D-[^{3}H]aspartate, and the next ten 2-min fractions for the

estimation of the evoked efflux of D-[^{3}H]aspartate. Taurine and other inhibitors were administered in the incubation medium and were already present from the beginning of superfusion. In some experimental groups THBA was loaded into the slices during the recovery and basal efflux periods and was excluded from incubation medium during the measurement of evoked release.

Based on the raw experimental data, two parameters of D-[^{3}H]aspartate efflux were calculated: the percentage of D-[^{3}H]aspartate released per minute and the fractional efflux rate constants. The total amount of D-[^{3}H]aspartate in a slice at the beginning of an experiment and the amount of label in the slice during each interval of fraction collection were calculated from the radioactivity in superfusate fractions and from the amount of label remaining in the slice at the end of the experiment.

The rate of D-[^{3}H]aspartate release during the period of fraction collection was computed as a percentage of the radioactivity released in one minute out of the total amount of label in the slice. This parameter shows the rate of D-[^{3}H]aspartate release during any short period of experiment.

The fractional efflux rate constants were calculated for longer experimental periods as the negative slopes for the regression lines of the logarithm of radioactivity remaining in a slice against the superfusion time. This parameter characterizes long-term changes in the efflux rate. The coefficient of correlation for the regression line, r, in all cases exceeded 0.95. The rate constants were determined for the prestimulation period (basal release, k_1) and for the stimulation period (evoked release, k_2). The period for calculation was selected on the basis of good linearity of the data.

Both efflux rates and efflux rate constants are presented as mean values ± SEM for four to twelve experiments. Statistical comparison was made using the two-tailed Student t-test. The level of statistical significance (p) was set at 0.05.

RESULTS

1. Amount and origin of basal taurine release (I, II)

The actual extracellular concentration of taurine in the rat striatum measured by in vivo microdialysis was estimated to be 25.2 ± 5.1 μM (I). The effect of pharmacological treatments on the extracellular taurine pool is summarized in Table 2. Perfusion of the competitive taurine transport inhibitor GES through the microdialysis probe significantly elevated the extracellular taurine concentration, while application of VSChC inhibitors SITS and DIDS reduced it more than twofold (I, II).

The classical inhibitors of neurotransmitter release TTX and Ca^{2+}-free medium affected likewise the basal release of taurine, their effects being however opposite to their classical effects on neurotransmitter release. Probe application of TTX led to a gradual increase in the interstitial taurine concentration in the striatum of anesthetized rats. Glutamate was also released in response to TTX, but the concentrations of threonine were not changed (I). Perfusion with Ca^{2+}-free aCSF during 140 min evoked a profound and stable increase in the taurine concentration in the dialysate (I, II). Omission of Ca^{2+} led to a small but statistically significant decrease in the interstitial glutamate concentration, while the threonine concentration was not affected (I). Ca^{2+}-free medium in combination with the calcium chelator EGTA had a more profound effect on taurine release than perfusion of Ca^{2+}-free medium alone (II). However, there were no statistically significant differences in the interstitial taurine concentrations under Ca^{2+} depletion with and without EGTA. To exclude the possible effect of altered ionic strength due to Ca^{2+} omission, Cl^- from $CaCl_2$ was substituted with equimolar concentrations of choline chloride. The magnitude of the evoked release of taurine was not altered (II).

Since Ca^{2+} omission displayed opposite effects on the release of taurine and glutamate, further studies of this phenomenon were required. In particular, we sought to determine the origin of the release and the involvement of voltage-sensitive calcium channels (VSCCs). The competitive inhibitor of TauT GES, preloaded into tissue, significantly diminished the Ca^{2+} omission-evoked release of taurine (II). However, in the presence of the VSChC inhibitor DIDS, Ca^{2+}-free medium evoked a significant release of taurine, the magnitude of which was comparable to that evoked by Ca^{2+}-free aCSF only (II). Both blockers of VSCCs Cd^{2+} and Ni^{2+} diminished the taurine release evoked by Ca^{2+} omission (II). Perfusion of Cd^{2+} reduced taurine concentrations to the basal level. However,

this level was still statistically different from the control values. Ni^{2+} was less effective, attenuating the effect of Ca^{2+}-free medium only during the first 60 min of application. In normal conditions inhibition of VSCCs by Ni^{2+} (a more potent blocker of P/Q VSCC channels) elevated release of taurine (II). Perfusion of Cd^{2+} (a more potent blocker of L/N VSCC channels) did not alter the interstitial concentrations of taurine.

Table 2. The effects of pharmacological treatment or ischemia on taurine release measured by microdialysis

Compound	Effect	Reference
GES	↓	I
SITS, DIDS	↑	I, II
TTX	↑	I
Ca^{2+}-free medium	↑	I, II
+ EGTA	no changes (versus Ca^{2+}-free medium)	II
+ GES	↓ (versus Ca^{2+}-free medium)	II
+ DIDS	no changes (versus Ca^{2+}-free medium)	II
+ Cd^{2+}	↓ (versus Ca^{2+}-free medium)	II
+ Ni^{2+}	↓ (versus Ca^{2+}-free medium)	II
Cd^{2+}	no changes	II
Ni^{2+}	↑	II
High K^{+} concentrations	↑	III
Ischemia	↑	III

2. Taurine release under ischemia and spreading depression (III)

SD evoked by 40 min application of aCSF containing 70 mM KCl through the microdialysis probe led to a marked increase in extracellular taurine in the striatum in vivo. The maximal increase was 8-fold when compared with the basal concentration of this amino acid (III). After the onset of control conditions taurine maintained high concentrations over a prolonged period, the level of taurine being still more than twofold higher than the basal level one hour after SD. At the same time SD led to a six-fold increase in the extracellular concentration of glutamate, which decreased immediately after the onset of control conditions. Ischemia evoked by bilateral occlusion of carotid arteries and systemic hypotension also induced a profound increase in the interstitial amounts of taurine in the striatum of rats in vivo. The maximal increase was 30-fold at 20 min after the onset of ischemia (III). During reperfusion, the taurine concentration still remained at the ischemic level after the first 20 minutes, approaching the basal level only one hour after the end of ischemia. Glutamate was also released during the ischemic conditions, but its extracellular concentration returned to basal level after 20 minutes of reperfusion.

3. The effect of taurine on D-[^{3}H]aspartate release (IV, V)

The inhibitory effects of taurine were studied in vitro using the efflux of D-[^{3}H]aspartate (a non-metabolized analog of glutamate) from superfused corticostriatal murine brain slices. The results of this study are summarized in Table 3. It was shown that chemical ischemia induced by sodium cyanide in KRH containing no glucose significantly enhanced the rate of D-[^{3}H]aspartate efflux (IV). Oxygen-glucose deprivation induced by incubating slices in glucose-free KRH under N_2 atmosphere also evoked D-[^{3}H]aspartate release, but less markedly (IV). Addition of 10 mM taurine to the medium at the beginning of superfusion did not alter the basal release rate or the fractional efflux rate constants of D-[^{3}H]aspartate efflux. However, taurine significantly reduced the release of D-[^{3}H]aspartate evoked by chemical ischemia or oxygen-glucose deprivation (IV). The lesser concentration of taurine (5 mM) did not influence the evoked release of D-[^{3}H]aspartate. The antagonists of GABA and glycine receptors bicuculline (0.1 mM) and strychnine (0.1 mM), respectively, did not affect the inhibitory effect of 10 mM taurine on D-[^{3}H]aspartate release induced by chemical ischemia (IV).

Incubation of corticostriatal slices with D-aspartate evoked D-[^{3}H]aspartate release (IV). Addition of the GluT competitive inhibitor THBA during the basal period of superfusion enhanced the efflux rate of D-[^{3}H]aspartate. However, the slices pretreated with THBA and subjected thereafter to D-aspartate in the absence of THBA released labeled D-aspartate at a slower rate than the slices incubated with D-aspartate but not preloaded with THBA (IV). Addition of 10 mM taurine to the incubation medium did not affect the efflux of D-[^{3}H]aspartate evoked by D-aspartate (IV). The efflux rate of D-[^{3}H]aspartate was also enhanced by incubation of corticostriatal slices in hypo-osmotic medium in which NaCl was reduced by 50 mM. The calculated osmolarity of this solution was 234 mOsm (calculated standard KRH osmolarity being 334 mOsm). The rate constants of D-[^{3}H]aspartate release from the slices incubated in hypo-osmotic medium were two times higher than the constants under basal conditions (IV). This effect was attenuated by the VSChC inhibitor SITS. However, the release of D-[^{3}H]aspartate evoked by the lowered osmolarity of the incubation medium was not attenuated by 10 mM taurine.

The activator of VSSCs veratridine induced a considerable increase in the efflux rate of D-[^{3}H]aspartate (IV, V). This increase was attenuated by removal of Ca^{2+} from the incubation medium and preloading of THBA into the slices. Taurine (10 mM) significantly diminished the veratridine-evoked release of D-[^{3}H]aspartate (IV, V). However, taurine did not inhibit veratridine-induced D-[^{3}H]aspartate release from slices preloaded with THBA or incubated in Ca^{2+}-free KRH (V). Other depolarizing agents, 50 mM KCl and the Na^+,K^+-ATPase inhibitor ouabain also evoked D-[^{3}H]aspartate release, but not so markedly as veratridine (V). Taurine did not attenuate the D-[^{3}H]aspartate release evoked by these agents (V).

Table 3. The effects of taurine on D-[^{3}H]aspartate release from superfused cortico-striatal slices in various conditions (main results)

Treatment	Effect	Reference
Chemical ischemia	↑↑↑	IV
+ taurine	↓↓ (versus chemical ischemia)	IV
+ taurine + bicuculline	↓↓ (versus chemical ischemia + bicuculline)	IV
+ taurine + strychnine	↓↓ (versus chemical ischemia + strychnine)	IV
Oxygen-glucose deprivation	↑↑↑	IV
+ taurine	↓↓ (versus oxygen-glucose deprivation)	IV
D-aspartate	↑	IV
+ taurine	no changes (versus D-aspartate)	IV
Hypo-osmotic medium	↑	IV
+ taurine	no changes (versus hypo-osmotic medium)	IV
Ouabain	↑	V
+ taurine	no changes (versus ouabain)	V
High K^+ concentrations	↑	V
+ taurine	no changes (versus high K^+)	V
Veratridine	↑↑↑	IV, V
+ taurine	↓↓ (versus veratridine)	IV, V
+ taurine + Ca^{2+}-free medium	no changes (versus veratridine + Ca^{2+}-free medium)	V
+ taurine + THBA	no changes (versus veratridine + THBA)	V
+ taurine + Cl^--free medium	↓ (versus veratridine + Cl^--free medium)	V
+ taurine + bicuculline	↓↓ (versus veratridine + bicuculline)	V
+ taurine + strychnine	↓↓ (versus veratridine + strychnine)	V
+ taurine + picrotoxin	no changes (versus veratridine + picrotoxin)	V
+ taurine + rotenone + oligomycin	↓ (versus veratridine + rotenone + oligomycin)	V
+ taurine + CCCP + oligomycin	no changes (versus veratridine + CCCP + oligomycin	V

Incubation of corticostriatal slices in the Cl^--free KRH led to an elevation of both basal and veratridine-evoked D-[^{3}H]aspartate release (V). Taurine reduced the veratridine-evoked release in the absence of Cl^-; however, its effect in these conditions was smaller compared to that in normal KRH (V). Nor did the antagonists of GABA and glycine receptors bicuculline and strychnine, respectively, at various concentrations (10 μM and 0.1 mM) affect veratridine-induced D-[^{3}H]aspartate release in the presence of taurine (V). However, the less specific inhibitor of both glycine and $GABA_A$ receptors picrotoxin (50 μM) completely prevented the inhibitory effect of taurine on veratridine-evoked D-[^{3}H]aspartate release (V). Higher concentrations of this compound (500 μM) were not effective in preventing the inhibitory effect of taurine (data not shown). The inhibitor of the mitochondrial electron transport chain rotenone (20 μM) or mitochondrial protonophore CCCP (2 μM) in combination with the inhibitor of mitochondrial ATPase oligomycin (15 μM) increased the release of D-[^{3}H]aspartate during both basal and evoked experimental periods (V). In the presence of taurine and under the inhibition of mitochondrial function, D-[^{3}H]aspartate release was slower eight minutes after veratridine administration, but the release at any other experimental time point was not affected (V). In the presence of CCCP taurine had no inhibitory effect on veratridine-evoked D-[^{3}H]aspartate release at any experimental time point.

DISCUSSION

1. Characteristics of taurine release under basal conditions

1.1. Magnitude of extracellular taurine pool

Taurine abounds in the brain of many mammalian species (Oja and Kontro, 1983; Huxtable, 1989). Its tissue concentration has been shown to be between 5-60 mmoles per kg of protein (Kreisman and Olson, 2003; Lima et al., 2004). Using the zero-net-flux approach, we showed now that under resting conditions the extracellular taurine concentration in the striatum amounts to 25.2 ± 5.1 µM (I). Our results correspond well to earlier data obtained for the rabbit olfactory bulb and rat dentate gyrus (Jacobson and Hamberger, 1985; Lerma et al., 1986). The physiological level of taurine in the extracellular space in these brain areas was shown to be between 8 and 20 µM. The values obtained for extracellular taurine concentrations appear to be quite high compared to the concentrations of other neurotransmitter amino acids. The extracellular glutamate concentration in the striatum has been shown to be around 1-3 µM (Miele et al., 1996; Lai et al., 2000), which is more than five times less than the estimated extracellular concentration of taurine in the same brain region. The extracellular concentrations of putative amino acid neurotransmitters in the dentate gyrus are also in the low micromolar range (0.8-2.9 µM, Lerma et al., 1986). However, the interstitial concentrations of the non-neuroactive amino acids are much higher than that of taurine, being in the high micromolar range. The intra/extracellular concentration ratios for these three groups are as follows: more than 2000 (putative neurotransmitters), less than 100 (non-neurotransmitter amino acids) and about 400 (taurine). Based on the size of the extracellular pool and the ratio of intra/extracellular concentrations, taurine occupies an intermediate position between neuroactive and non-neuroactive compounds.

1.2. Uptake and release of taurine under resting conditions

Due to its hydrophilic molecular structure, taurine poorly penetrates the lipid bilayer of the plasma membrane. By reason of the impossibility of physical diffusion, the extracellular taurine pool in neural cells is probably regulated by specific mechanisms, which include release and uptake. Uptake of taurine is accomplished by the active sodium-mediated transport systems. Using the taurine transport inhibitor GES we demonstrated active uptake of taurine under

resting conditions in vivo (I). Application of 1 mM GES significantly elevates the basal extracellular taurine concentrations. The active taurine uptake under resting conditions in vivo is similar to the uptake of other neurotransmitter amino acids. For example, the basal concentration of glutamate is elevated by inhibition of transport systems (Rawls and McGinty, 1997; Janáky et al., 2001).

The evoked release of taurine from neural tissue could be mediated by several mechanisms, including leakage through membrane channels, exocytosis of vesicular taurine from nerve endings, and carrier-mediated transport (Oja and Saransaari, 2000). Taurine release evoked by ischemia (Phillis et al., 1997; Saransaari and Oja, 1998) and hypo-osmotic stimuli (Pasantes-Morales et al., 1990; Estevez et al., 1999) is partially mediated by VSChCs. Now it was shown that the VSChC inhibitors SITS and DIDS diminish the extracellular taurine concentration in vivo under resting conditions (I, II). We infer from the data obtained that the extracellular taurine pool is supplied by the exit of taurine through volume-sensitive anion channels and that about one half of taurine exits through these channels under resting conditions.

1.3. Release of taurine evoked by inhibition of synaptic exocytosis

Data on the synaptic release of taurine remain contradictory. The synaptosomal fraction is highly enriched by taurine (Kontro et al., 1980). However, active uptake of taurine into the synaptic vesicles has not been shown (Fyske and Fonnum, 1996). Taurine is released from nervous tissue in response to depolarizing stimuli, e.g., increased extracellular K^+ concentrations, veratridine and agonists of glutamate receptors (III, Kontro and Oja, 1987d; Saransansaari and Oja, 1997, 1999c, 2003). This depolarization-evoked release is apparently consistent with the exocytotic liberation of taurine from neural preparations. To ascertain the involvement of synaptic mechanisms in the basal release of taurine, we now estimated the effect of inhibition of voltage-sensitive sodium channels by TTX and the reduction of the Ca^{2+} uptake by Ca^{2+}-free medium on the basal taurine release (I). The release of glutamate was used in these experiments as a positive, and that of threonine as a negative reference.

1.3.1. Effect of tetrodotoxin application

It was now shown that the extracellular concentrations of both glutamate and taurine are increased by TTX application, while threonine concentrations remain unaltered (I). Previous results on TTX effects on taurine and glutamate release are discrepant. For instance, the basal release of taurine in the substantia nigra in vivo and from brain slices in vitro has been slightly reduced or unaffected by inhibition of voltage-gated Na^+ channels by TTX (Biggs et al., 1995; Bianchi et al., 1999). The kainate-stimulated taurine release was reported to be insensitive to inhibition of VSSCs (Bianchi et al., 1998). In several studies no changes or a minor decrease in glutamate concentration have been detected in response to inhibition of the voltage-gated sodium channels (for a review see Timmerman

and Westerink, 1997). However, in those studies specifically conducted on the striatum in vivo, extracellular glutamate was increased under TTX application (Keefe et al., 1993; Morari et al., 1996). This phenomenon may reflect TTX-mediated disinhibition of cortical glutamatergic terminals. The firing of cortical and thalamic motoneurons is controlled by the striato-thalamo-cortical loop (Parent and Hazrati, 1995a,b). Under resting conditions the striatal efferents are silent, leaving thalamic neurons suppressed. The dopaminergic striatonigral projections, which are tonically active, balance the activity of the two branches of the loop. Intrastriatal application of TTX attenuates dopaminergic tone and leads to disinhibition and subsequent release of glutamate from the cortical inputs. In our studies taurine was released upon TTX application in a similar manner and in the same order of magnitude as glutamate (I). Taurine is thus either released from the same site (cortical inputs) or glutamate induces taurine release from other sources. It has previously been reported that decortication reduces the striatal levels of glutamate, but leaves the taurine concentrations unchanged (Butcher and Hamberger, 1987). The TTX-induced taurine elevation is thus likely to occur secondarily after glutamate release. Although taurine may be released by a non-synaptic mechanism, the results obtained show that release is strongly coupled to changes in neuronal activity.

1.3.2. Effect of Ca^{2+}-free medium

Depolarization-evoked taurine release has been shown to be partially Ca^{2+}-dependent, suggesting the involvement of the exocytotic pathway (Kamisaki et al., 1993; Menéndez et al., 1993). However, some investigators have failed to show any Ca^{2+} dependency of evoked taurine release (Holopainen et al., 1985; Solís et al., 1986). The main factor hampering definitive conclusions on the Ca^{2+} dependency is that Ca^{2+} depletion significantly enhanced the basal release of taurine in the studies in question. Nevertheless, when K^+-stimulated release under Ca^{2+}-free conditions is compared to the basal release (Ca^{2+}-free conditions only), a significant reduction in the evoked release in the absence of Ca^{2+} is evident (Korpi and Oja, 1984; Oja et al., 1985; Oja and Kontro, 1987).

The basal taurine release, measured in the absence of any stimuli, was here enhanced in extracellular Ca^{2+}-free medium (I, II). Using superfused brain slices and astrocytes in culture, it has been shown in vitro that Ca^{2+}-free medium elevates extracellular taurine and that Ca^{2+} chelators enhance this effect (Korpi and Oja, 1984; Oja et al., 1985; Saransaari and Oja 1992; Takuma et al., 1996). We confirmed these data using in vivo microdialysis (II). The observed effect is specific for taurine; omission of Ca^{2+} reduced extracellular glutamate concentrations and did not change extracellular threonine concentrations.

Due to methodological problems it is difficult to prove the Ca^{2+} dependency of the release measured by microdialysis. The basal glutamate release has been found to be unchanged or only slightly decreased after omission of Ca^{2+} (for review see Timmerman and Westerink, 1997). We showed a minor but statistically significant decrease in extracellular glutamate, which was likewise

discernible in Ca^{2+}-free medium. In contrast, taurine release was significantly enhanced in the absence of Ca^{2+}. Interestingly, the omission of Ca^{2+} has no effect on taurine release in the supraoptic nucleus of the hypothalamus, where taurine is localized mainly in glia (Decavel and Hatton, 1995; Hussy et al., 2000). Since the localization of taurine in the striatum is predominantly neuronal (Storm-Mathisen and Ottersen, 1986; Madsen et al., 1987; Della Corte et al., 1990), the enhanced taurine release in Ca^{2+}-free solution may be a phenomenon specific to neurons but not glia.

Summarizing, inhibition of synaptic transmission by the voltage-sensitive Na^+ channel inhibitor tetrodotoxin and Ca^{2+} depletion results in an increase in extracellular taurine, suggesting that synaptic exocytosis is not involved in the regulation of the extracellular pool of this amino acid under basal conditions. These results would indicate that taurine release is related to neuronal activity, although in a non-classical manner. However, the results obtained do not exclude the involvement of synaptic mechanisms in the stimulated taurine release.

1.3.3. Mechanisms of taurine release evoked by Ca^{2+}-free medium

The effect of Ca^{2+} on the extracellular taurine concentrations is of special interest in the context of the regulatory mechanisms of taurine release. We now characterized the basal release of taurine in Ca^{2+}-free medium in the rat striatum in vivo and determined the driving force underlying the enhancement of release of taurine under these conditions (II).

Taurine can be released by a number of pathways, two of them being the most prominent under normal conditions: release through volume-sensitive chloride channels and the reverse action of TauT. Both modes may be responsible for the elevated efflux of taurine under Ca^{2+} depletion. However, the upregulation of VSChCs under hypo-osmotic conditions has been shown to be essentially Ca^{2+}-independent (Pasantes-Morales and Morales Mulia, 2000). Takuma and associates (1996) have demonstrated that Ca^{2+} depletion releases taurine through volume-independent mechanisms, since Ca^{2+}-free medium does not cause any cell swelling. Furthermore, the K^+/Cl^- co-transport inhibitor furosemide and sucrose do not affect taurine release under Ca^{2+} depletion. In our study, in the presence of the VSChC inhibitor DIDS, omission of Ca^{2+} evoked the release of taurine in the same magnitude when compared to the release in Ca^{2+}-free aCSF alone (II). These data confirm the conclusion of Takuma and associates (1996) that osmoregulatory mechanisms are not involved in the phenomenon in question.

Since Ca^{2+} depletion alters the membrane gradient of Na^+ ions, the efflux of neurotransmitters in the absence of calcium can be mediated by transport systems operating in a reverse mode (Bernath, 1992). Takuma and colleagues (1996) were the first to propose that TauT provides for the exit of taurine from cells in Ca^{2+}-free medium. To test whether the TauT pathway of taurine release

is involved under Ca^{2+}-free conditions, we inhibited its reverse action by GES loaded into the cells. After a one-hour application of GES, Ca^{2+}-free medium did not evoke any significant elevation in the interstitial taurine concentration (II). These data confirm that Ca^{2+} depletion reverses TauT and enhances taurine release from the cells.

The Ca^{2+} dependency of neurotransmitter release is based on the inhibition of Ca^{2+} entry via VSCCs and a subsequent decrease in $[Ca^{2+}]_i$ (Berridge et al., 2003). We tested whether the inhibition of Ca^{2+} entrance through VSCCs underlies the enhancement of taurine release in the absence of Ca^{2+}. Both Cd^{2+} and Ni^{2+} at micromolar concentrations are potent inhibitors of VSCCs (Yamakage and Namiki, 2002). Cd^{2+} has a higher affinity for the L/N types of VSCCs, while Ni^{2+} is a more potent inhibitor of the P/Q channels. Blocking of the VSCCs of P/Q type in the presence of extracellular Ca^{2+} elevated the extracellular taurine concentration (II). Since direct VSCC inhibition mimics the effect of Ca^{2+} omission from the extracellular space, we conclude that a part of the taurine release evoked by Ca^{2+}-free medium is due to a decrease in Ca^{2+} flow through the VSCCs of P/Q type.

We have now shown that the inhibition of VSCCs evokes taurine release. One could thus expect VSCC blockers to enhance the effect of Ca^{2+}-free medium. On the contrary, both $CdCl_2$ and $NiCl_2$ attenuated the effect of Ca^{2+} omission (II). These data are in accord with previous findings. In particular, the VSCC inhibitor nifedipine has been shown to attenuate the Ca^{2+} depletion-evoked release of taurine from astrocytes (Takuma et al., 1996).

The selectivity of VSCCs for Ca^{2+} is particularly high. It binds to the pore walls and the influx of this ion through VSCCs is highly specific (Sather and McCleskey, 2003). The presence of Ca^{2+} inside the pore eliminates nonspecific flow through VSCCs. However, other ions of smaller size are able to penetrate VSCCs in the absence of Ca^{2+}. Under these conditions a fast but non-specific flow of Na^+ through VSCCs has been described (Almers et al., 1984; Fukushima and Hagiwara, 1985). We suggest that under calcium depletion this nonspecific efflux of Na^+ disrupts the membrane gradient of sodium ions, reverses TauT and thus initiates the release of taurine.

2. Taurine release under ischemia and spreading depression

We showed that taurine is released abundantly during ischemia and SD and still remains elevated during reperfusion (III). Its maximal release was detected at the end of ischemic conditions, and the first 20 minutes of reperfusion did not change the level of release. Our results are in accord with those of earlier studies (Baker et al., 1991; Obrenovitch et al., 1993). Such delayed responses are characteristic of this amino acid both in vitro (Saransaari and Oja; 1992) and in vivo (Scheller et al., 2000a). The increased extracellular contents of this amino acid may reflect compensatory mechanisms activated in response to injury. In

particular, the late peak release of taurine and the slow recovery after ischemic and depolarizing conditions reflect this function.

As discussed above, the extracellular concentration of taurine in the brain in vivo is about ten times higher than the concentrations of established amino acid neurotransmitters (I, Lerma et al., 1986; Miele et al., 1996). The concentration of taurine in the synaptic cleft is probably still much higher than the overall extracellular concentration estimated by microdialysis. Ischemia evokes an approximately 30-fold increase in extracellular taurine concentration in vivo (III). This means that the actual extracellular concentration under these conditions may reach at least the one-mM range. In keeping with this, relatively high concentrations of taurine have been found to be effective in other studies on different brain preparations (e.g., del Olmo et al., 2000b; Messina and Dawson, 2000; El Idrissi and Trenkner, 2003).

3. Inhibitory effect of taurine on the release of glutamate

3.1. Effect of taurine on various types of glutamate release

3.1.1. Effect on ischemia-evoked D-[^{3}H]aspartate release

The effect of exogenously applied taurine on glutamate release was studied using D-[^{3}H]aspartate, which is widely employed as a marker for L-glutamate release (Rutledge and Kimelberg, 1996; Roettger and Lipton, 1996; Saransaari and Oja, 1999d; Dohovics at el., 2003; Marcoli et al., 2003). D-[^{3}H]Aspartate has been claimed to be taken up by glial cells and nerve terminals but not by synaptic vesicles (Gundersen et al., 1995). However, other biochemical studies suggest that this analog can be used as a marker for both cytoplasmic and vesicular pools of glutamate. In particular, D-[^{3}H]aspartate has been shown to be released from synaptic terminals by high-potassium-evoked depolarization, NMDA application and electric stimulation in a Ca^{2+}-dependent manner (Muzzolini et al., 1997; Savage et al., 2001; Bak et al., 2003). The release of glutamate and D-[^{3}H]aspartate from the cytosolic pool occurs in a similar manner (Roettger and Lipton, 1996; Saransaari and Oja, 1999d). These findings validate the use of D-[^{3}H]aspartate as an indicator of glutamate release under most experimental conditions.

To study the effect of taurine on ischemia-evoked glutamate release, two models of in vitro ischemia were used. Both experimental conditions inhibit glycolysis and oxidative metabolism and lead to excitotoxic cell death mediated by overactivation of glutamate receptors (Vornov, 1995). Chemical ischemia and oxygen-glucose deprivation induced a marked release of D-[^{3}H]aspartate, which was significantly reduced by 10 mM taurine (IV). Taurine has also inhibited the release of neurotransmitters, in particular that of glutamate (Kamisaki et al., 1993; Zheng, 2001).

Glutamate is released by several mechanisms during ischemia (Phillis and O’Regan, 2003). In the first minutes of ischemia the energy sources are still not

fully exhausted and glutamate release is activated by means of synaptic vesicular mechanisms triggered by membrane depolarization (Wahl et al., 1994). After total energy depletion, the subsequent efflux includes non-vesicular Ca^{2+}-independent release (Seki et al., 1999). It is mediated in part by Na^{+}-dependent amino acid transporters at plasma membranes operating in a reversed mode due to the ischemia-evoked ionic disbalance. The other non-vesicular glutamate release under ischemia is mediated by the swelling-induced opening of chloride channels. The above three means of glutamate release under ischemia have also been shown for D-[^{3}H]aspartate release (Roettger and Lipton, 1996; Saransaari and Oja, 1999d; Marcoli et al., 2003). To determine which mechanism of ischemic D-[^{3}H]aspartate release is affected by taurine, the different modes of release were now modeled pharmacologically.

3.1.2. Effect on D-[^{3}H]aspartate release under hypo-osmotic conditions

Under hypo-osmotic conditions cells swell and regulate their volume by extruding osmotically active substances. The amino acids released during this regulatory volume decrease include aspartate, taurine, glutamate, GABA and glycine (Estevez et al., 1999; Phillis and O'Regan, 2003). Of these, taurine is considered the most efficacious osmolyte (Schaffer et al., 2000) and application of exogenous taurine has been shown to enhance cell volume regulation (Kreisman and Olson, 2003). We showed now that D-[^{3}H]aspartate is likewise released under hypo-osmotic conditions and that the inhibitor of volume-activated chloride channels SITS is able to attenuate this release (IV). However, we failed to show any effect of taurine on the hypo-osmotically evoked D-[^{3}H]aspartate release, witnessing that the inhibitory effect of taurine on the ischemia-evoked D-[^{3}H]aspartate release is not likely to be mediated by volume-regulatory mechanisms.

3.1.3. Effect on transporter-mediated D-[^{3}H]aspartate release

Glutamate is taken up into cells by means of GluT, a family of proteins which mediate the glutamate flux driven by sodium ion gradients (Nelson, 1998). In ischemia, due to energy failure, the ionic gradient is dissipated and the direction of action of amino acid transporters may be altered. It has been shown that the glutamate carrier blocker dihydrokainate suppresses glutamate release under global cerebral ischemia (Seki et al., 1999). To model this mode of glutamate release we used the phenomenon of homoexchange, when exogeneous application of the compound evokes the increase in extracellular concentration of the same compound preloaded into the tissue. Under these conditions, an extracellular compound both inhibits the reuptake and evokes the release of the preloaded substance (Bernath, 1992). In our experiments D-[^{3}H]aspartate release was enhanced in the presence of unlabeled D-aspartate in the incubation medium (IV). Uptake blockers can be used to prove the involvement of amino acid carriers in the release during homoexchange (Bernath, 1992). The participation of the GluT in homoexchange-induced release was confirmed

using the competitive glutamate transport inhibitor THBA. Being a a competitive inhibitor, THBA needs to act from the same site as the intracellularly loaded D-[^{3}H]aspartate. Preloading of the competitive inhibitor before application of a stimulus will inhibit the release mediated by the reversed action of a transporter (Rutledge and Kimelberg, 1996). Moreover, to distinguish between the inhibition of reuptake and activation of release mediated by the transporter due to homoexchange, the inhibitor of the carrier should be present only intracellularly. We showed here that THBA increases the basal extracellular concentration of D-[^{3}H]aspartate, probably by heteroexchange. THBA preloaded into the slices during the basal period of reperfusion and removed during the stimulation period significantly inhibited the release of D-[^{3}H]aspartate evoked by homoexchange (IV). This confirms that D-[^{3}H]aspartate release evoked by homoexchange is mediated by GluT. However, taurine did not interfere with this mode of D-[^{3}H]aspartate release.

3.1.4. Effect on depolarization-evoked D-[^{3}H]aspartate release

Since taurine does not interact with the non-vesicular release of D-[^{3}H]aspartate evoked either by the reverse action of the transporters or by the opening of volume-activated chloride channels, one may conclude that the possible target of taurine action is the synaptic release. To test this alternative, study was made of the effect of taurine on the D-[^{3}H]aspartate release evoked by depolarization using the activator of VSSCs veratridine. This compound evoked a marked release of D-[^{3}H]aspartate, which was reduced under Ca^{2+}-free conditions and by THBA preloaded into the slices. Omission of Ca^{2+} from the incubation medium inhibits the synaptic release pathway. Preloading of the glutamate transport inhibitor THBA suppresses the glutamate release mediated by the reversal of carriers evoked by the disturbed membrane gradient of Na^+. We have now shown that both Ca^{2+}-free medium and THBA preloaded into the slices suppress veratridine-evoked D-[^{3}H]aspartate release, signifying that veratridine-evoked depolarization leads to glutamate release through both pathways (IV, V).

Taurine significantly reduced the release of D-[^{3}H]aspartate evoked by this mode. In the presence of THBA inside the slices or under Ca^{2+} depletion taurine did not now affect veratridine-evoked D-[^{3}H]aspartate release. When the release by synaptic exocytosis or by the reverse action of GluT is blocked, taurine does not thus inhibit the remaining D-[^{3}H]aspartate release**,** indicating that it inhibits the depolarization-evoked glutamate release mediated by both release pathways. However, we were able to show that taurine does not directly influence the release evoked by homoexchange. Our data thus indicate that the target of taurine effect may be a molecular event lying upstream of the glutamate release reactions. As depolarization is the common activation factor for the release of glutamate through both discussed pathways, we may to assume that taurine reduces depolarization, acting before the particular mechanisms of glutamate release. This hypothesis is in accord with the findings of Wu and associates

(2005), who have shown that taurine reduces glutamate-evoked Ca^{2+} influx and that this effect is non-specific and independent of the type of Ca^{2+} channel. Taurine may thus hyperpolarize cell membranes and affect voltage-dependent Ca^{2+} influx into the cell. Such a conception is corroborated by the finding that taurine indeed impedes glutamate-induced membrane depolarization (Wu et al., 2005).

The effect of taurine is specific to the depolarization-induced release evoked by veratridine. Taurine did not affect the D-[^{3}H]aspartate release evoked by high K^+ concentrations and the Na^+, K^+-ATPase inhibitor ouabain. A link between the putative taurine molecular target and voltage-gated sodium channels may thus exist. Taurine alters the properties of tetrodotoxin-sensitive Na^+ currents in rat dorsal root ganglion neurons (Yu et al., 2005). This effect may be related to the action of taurine as the charged substance on the lipid surroundings of the Na^+ channels or to interactions with a site allosterically coupled to the Na^+ channel subunits. Taurine may also influence the state of membrane polarization, increasing membrane Cl^- conductance (Oja et al., 1990; Belluzzi et al., 2004; El Idrissi and Trenkner, 2004) and thus antagonize the depolarization evoked by activation of Na^+ channels.

3.2 Molecular mechanisms of the effect of taurine

3.2.1. Role of agonist-gated chloride channels

During the previous step, it was shown that taurine affects the depolarization-evoked component of ischemic glutamate release. To elucidate the mechanisms of this effect we studied the involvement of agonist-gated chloride channels, in particular $GABA_A$ and glycine receptors, as well as the Ca^{2+} uptake by mitochondria.

Since taurine induces chloride currents across cell membranes (Oja et al., 1990; Belluzzi et al., 2004), the main molecular target of its action could be a chloride channel. To verify this hypothesis, we studied the effect of Cl^--free superfusion medium on the inhibitory effect of taurine. While Cl^- omission enhanced both basal and veratridine-evoked D-[^{3}H]aspartate release, the effect of taurine under these conditions was reduced. This would confirm the involvement of some Cl^- channels in the mechanism of the inhibitory effect of taurine. Some taurine actions on neuronal preparations may be mediated by $GABA_A$ and glycine receptors (Malminen and Kontro, 1986; Kontro and Oja, 1987b; Ye et al., 1997; O'Burne and Tipton, 2000, Belluzzi et al., 2004; Louzada et al., 2004). However, the specific inhibitors of $GABA_A$ and glycine receptors, bicuculline and strychnine, respectively, did not alter the effect of taurine on ischemia- or veratridine-evoked D-[^{3}H]aspartate release. The effects of taurine observed in this study are thus not likely to be mediated by the GABA- or glycine-governed chloride channels.

However, the less specific inhibitor of these receptors picrotoxin at a concentration of 50 μM completely prevented the taurine effect. Similar effects of Cl^- channel inhibitors have been reported in other studies. In an in vivo model of ammonia neurotoxicity, picrotoxin significantly reduced the protective effect of taurine, while bicuculline and strychnine were less effective (Hilgier et al., 2005). Vasopressin release induced by taurine from the rat neurohypophysis is blocked by picrotoxin, but not by bicuculline or strychnine (Song and Hatton, 2003). The convulsant alkaloid picrotoxin is an equimolar mixture of picrotoxinin and picrotin, and is regarded as a channel blocker for $GABA_A$ receptors, $GABA_C$ receptors and α-homomeric glycine receptors (Jentsch et al., 2002; Lynch, 2004). Since the selective antagonists of $GABA_A$ and glycine receptors were shown here to be ineffective in preventing the inhibitory effect of taurine, the effect of picrotoxin is not related to the inhibition of these types of chloride channels. $GABA_C$ receptors are also sensitive to picrotoxin but practically not gated by taurine (Jentsch et al., 2002; Martínez-Torres and Miledi, 2004), rendering their involvement in the inhibitory effect of taurine questionable. The ability of picrotoxin to prevent the suppressive effect of taurine on the evoked D-[^{3}H]aspartate release may be related to the activation of the putative taurine receptor. If this is so, then the putative taurine receptor should be deprived of the binding sites for bicuculline and strychnine but possess the picrotoxin-binding site. Picrotoxin sensitivity is apparently determined by the presence of threonine in 6' position in the second transmembrane domain, which forms pore walls in $GABA_A$ and glycine α subunits (Lynch, 2004). It is possible to suggest that the structure of the taurine receptor may be very similar to that of GABA and glycine-gated chloride channels, with maximal homology in the protein domains which form the channel pore. It is interesting to note that while taurine usually demonstrates low affinity for the native GABA or glycine receptors, certain point mutations substantially increase its potency to activate the channels associated with these receptors (Martínez-Torres and Miledi, 2004; Miller et al., 2004).

3.2.2. Role of mitochondria

Taurine is also thought to exert its action through regulation of $[Ca^{2+}]_i$ by affecting Ca^{2+} storage pools. Taurine activates the accumulation of $^{45}Ca^{2+}$ in mitochondria isolated from rat liver or cortical nerve endings (Li and Lombardini, 1991; Palmi et al., 1999). This effect appears to be related to the action of taurine on the mitochondrial uniporter, since taurine does not alter the Ca^{2+} release evoked by ruthenium red (Palmi et al., 1999) and does not prevent the opening of permeability transition (Palmi et al., 2000). The mitochondrial Ca^{2+} uptake is mediated by the mitochondrial uniporter and driven by the membrane potential (Duchen, 1999; Nicholls and Budd, 2000). One of the experimental approaches to inhibit the action of the mitochondrial uniporter includes the use of mitochondrial inhibitors, which induce a collapse of the mitochondrial membrane potential (Khodorov et al., 1999; Billups and Forsythe,

2002). To examine the involvement of mitochondrial Ca^{2+} uptake in the inhibitory effects of taurine on the evoked D-[^{3}H]aspartate release, we blocked this uptake by either the respiratory chain blocker rotenone or the mitochondrial protonophore CCCP in combination with the mitochondrial ATPase inhibitor oligomycin. The latter prevents the fast ATP depletion caused by the reversed operation of the mitochondrial ATP synthase. Rotenone and oligomycin have been shown to prevent Ca^{2+} uptake by mitochondria under excitotoxic conditions and after short-term depolarization (Khodorov et al., 1999; Billups and Forsythe, 2002). CCCP in combination with oligomycin also prevents Ca^{2+} clearance by mitochondria from the intracellular space (Babcock and Hille, 1998; Dykens et al., 2002). In our study, they enhanced D-[^{3}H]aspartate release under both basal conditions and during veratridine administration. However, in the presence of these inhibitors the suppressive effect of taurine on D-[^{3}H]aspartate release was significantly diminished or absent. These results bespeak the involvement of regulation of cellular Ca^{2+} homeostasis in the mechanisms underlying the taurine-induced inhibition of glutamate release. However, the question whether the activation of picrotoxin-dependent chloride fluxes and mitochondrial Ca^{2+} regulation are parts of a single chain of taurine-activated molecular reactions or are independent sites of action remains open.

SUMMARY OF RESULTS

The main findings of the study were:

1) The extracellular pool of taurine in the rat striatum under resting conditions in vivo amounts to 25 μM. It is reduced by the inhibitors of volume-sensitive chloride channels and elevated by the inhibitor of the taurine transporter.
2) The inhibition of neuronal activity by the reduction of extracellular Ca^{2+} concentrations and inhibition of voltage-gated sodium and calcium channels elevates the extracellular taurine in the striatum in vivo. Taurine release under Ca^{2+} omission is reduced by the inhibitor of the taurine transporter and voltage-gated calcium channels.
3) Taurine is released abundantly in response to physiological (SD) and pathological (ischemia) stimuli. However, the extracellular concentrations of taurine slowly return to the basal level after the onset of control conditions.
4) Exogeneous taurine suppresses the release of preloaded D-[^{3}H]aspartate, evoked by ischemia or veratridine-induced depolarization. At the same time, taurine has no effect on the release evoked by unlabeled D-aspartate, hypo-osmotic conditions, high K^+ concentrations and ouabain.
5) Both Cl^- omission from the incubation medium and inhibition of the mitochondrial Ca^{2+} uptake reduced the effect of taurine on veratridine-evoked D-[^{3}H]aspartate release. The specific antagonists of $GABA_A$ and glycine receptors bicuculline and strychnine were ineffective, while the less specific ligand-gated Cl^- channel blocker picrotoxin completely prevented the inhibitory taurine effect.

CONCLUSIONS

The main aim of the study was to characterize taurine as a neuromodulator in the corticostriatal system of the rodent brain. To this end we described the magnitude of the extracellular taurine pool, the mechanisms which regulate it and the mode and mechanisms of the effect of taurine on glutamate release.

It was shown that the extracellular taurine pool in the rat striatum in vivo is large compared to neurotransmitter amino acids. Under resting conditions it is dynamically regulated by the exit of taurine through volume-sensitive anion channels and uptake by taurine transporter.

Basal taurine release does not occur via the synaptic release pathway. Moreover, the inhibition of synaptic transmission by reduction of extracellular Ca^{2+} concentrations and inhibition of voltage-sensitive sodium and calcium channels does not reduce, but elevates the level of extracellular taurine in vivo. Omission of Ca^{2+} ions enhances the release of taurine through the reversal of taurine transporter. This evidence shows that although taurine is not released by a synaptic mechanism, its release is strongly coupled to changes in neuronal activity produced by inhibition of synaptic transmission.

Ischemia and spreading depression evoke a profound release of taurine. The extracellular amount of taurine may be elevated 30-fold under these conditions and the actual extracellular concentration of taurine may reach at least one-mM range. We thus confirmed that the high concentrations of taurine necessary to obtain its inhibitory effect may be present in the rat brain in vivo.

Taurine suppresses the release of glutamate evoked by ischemia or depolarization. However, it did not change hypo-osmotic or transporter-mediated glutamate release. The effect of taurine may be mediated by an agonist-gated chloride channel, but not by $GABA_A$ or glycine receptors, and by the regulation of Ca^{2+} sequestration by mitochondria.

To summarize, it was proved that the extracellular taurine pool in the striatum is large and strictly regulated. Extracellular taurine is affected by changes in neuronal activity, allowing taurine to fulfil neuromodulatory functions. Taurine may suppress glutamate release through regulation of membrane polarization and intracellular concentrations of Ca^{2+}. Overall, the findings in this study confirm the role of taurine as an inhibitory neuromodulator in the corticostriatal system of the rodent brain and shed light on the possible mechanisms of its neuromodulatory effects.

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Characteristics of basal taurine release in the rat striatum measured by microdialysis

S. Molchanova[1,2], **S. S. Oja**[1,3], and **P. Saransaari**[1]

[1] Brain Research Center, Medical School, University of Tampere, Tampere, Finland
[2] I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry, Saint Petersburg, Russia
[3] Department of Clinical Physiology, Tampere University Hospital, Tampere, Finland

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Summary. Taurine is a sulfur-containing amino acid thought to be an osmoregulator, neurotransmitter or neuromodulator in the brain. Our objective was to establish how much taurine is released in the striatum and examine the mechanisms controlling extracellular taurine concentrations under resting conditions. The experiments were made on rats by microdialysis *in vivo*. Changes in taurine were compared with those in glutamate, glycine and the non-neuroactive amino acid threonine. Using the zero net flux approach we showed the extracellular concentration of taurine to be 25.2 ± 5.1 μM. Glutamate was increased by tetrodotoxin and decreased by Ca^{2+} omission, glycine and threonine were not affected and both treatments increased extracellular taurine. The basal taurine release was increased by the taurine transport inhibitor guanidinoethanesulfonate and reduced by the anion channel blocker 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

Keywords: Taurine – Glutamate – Glycine – Threonine – Zero net flux – Synaptic transmission

Introduction

Taurine (2-aminoethanesulphonic acid) is a non-essential amino acid abundant in the central nervous system (CNS) of mammals. It is released from nervous tissue by hypoosmotic stimuli and regulates cell volumes (Oja and Saransaari, 1996; Hussy et al., 2000; Pasantes-Morales et al., 2002; Kreisman and Olson, 2003). However, taurine is highly heterogeneously distributed in the brain (Kontro et al., 1980; Palkovits et al., 1986; Pow et al., 2002). It may therefore serve other functions aside from osmoregulation, being specific for different brain regions. Taurine has been thought to be a neuromodulator or transmitter in the brain (Oja and Kontro, 1983). Indeed, many characteristics of taurine release, uptake and neuronal effect correspond well to those of neurotransmitters. Application of taurine generally modifies neuronal electrical potentials by increasing Cl^- conductance and evokes hyperpolarization (Oja et al., 1990; Belluzzi et al., 2004). In the striatum it also potentiates synaptic transmission (Chepkova et al., 2002). Taurine is released from nervous tissue in response to depolarizing agents such as N-methyl-D-aspartate (NMDA) and high concentrations of K^+ (Kontro and Oja, 1987d; Menendez et al., 1993; Semba et al., 1995; Saransaari and Oja, 1991, 1997, 2003), and sequestered by active high-affinity uptake systems (Kontro and Oja, 1978; Oja and Kontro, 1984). Extracellular taurine modifies the release of amino acid transmitters and modulates intracellular Ca^{2+} homeostasis (Kamisaki et al., 1993; Foos and Wu, 2002).

Many specific questions, however, remain open, hampering clear conclusions as to the neurotransmitter or neuromodulatory functions of taurine. In first place, the target of taurine action in the brain has not been defined. Many neuronal effects of taurine are diminished by GABA and glycine receptor antagonists (Malminen and Kontro, 1986; Kontro and Oja, 1987c; Ye et al., 1997; Chepkova et al., 2002; Belluzzi et al., 2004). A specific taurine receptor in the synaptic membranes has been described (Kontro and Oja, 1983, 1987a, 1987b; Frosini et al., 2003), but some investigators have failed to demonstrate it (Lähdesmäki et al., 1977; Lopez-Colomé and Pasantes-Morales, 1981). The origin of taurine release is also not precisely known. Many studies have failed to witness the neuronal origin of the K^+- or NMDA-evoked taurine release (Hanretta and Lombardini, 1987; Semba et al.,

1995). Taurine release is invariably slower in onset and offset (Korpi et al., 1981; Kontro and Oja, 1987d). The data concerning the accumulation of taurine in synaptic vesicles are somewhat contradictory (Kontro et al., 1980; Fyske and Fonnum, 1996). The conclusion must be that the mechanisms of taurine release are not necessarily the same as those of classical neurotransmitters.

One approach to make this situation clearer is to describe taurine release under resting conditions and to compare the data to the characteristics of neurotransmitter release. The aim of our study was thus to assess the order of magnitude of basal taurine release in the rat striatum *in vivo* and to characterize the regulating mechanisms. The results obtained were compared with the characteristics of glutamate release. The zero net flux approach was adopted to determine the extracellular taurine concentration. We also studied whether basal taurine release is regulated by the taurine uptake systems and which mechanisms are involved in maintaining basal release. The taurine transport inhibitor guanidinoethanesulfonate (GES) was used to evaluate whether taurine uptake is active. To ascertain the synaptic origin of taurine release, we tested how the release is affected by tetrodotoxin (TTX) and Ca^{2+} omission. Changes in the taurine concentration were compared with those of the neurotransmitter glutamate and the non-neuroactive amino acid threonine. In order to determine whether or not glycine acts as a neurotransmitter in the striatum, changes in its concentrations were also monitored. Since taurine release may greatly contribute to cell volume regulation, we also tested how the basal extracellular taurine level is affected by application of the anion channel blocker 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS).

Materials and methods

Animals

The studies were carried out on anesthetized adult Sprague-Dawley male rats (250–300 g weight). The experimental procedures were in accordance with the European Community Directive for the ethical use of experimental animals. All efforts were made to minimize both the suffering and the number of animals used.

Microdialysis surgery

Implantation of microdialysis probes was done on the day of the experiment under halothane narcosis. The animals were first anesthetized with 4% halothane in air and placed in a stereotaxic frame (David Kopf Instruments, Düsseldorf, Germany). The concentration of halothane was then reduced to 0.8–1.2% and body temperature kept at 37.5°C with a heating pad. Microdialysis probes of concentric design (4 mm length, 0.5 mm o.d.) were implanted in the striatum. The stereotaxic coordinates were as follows: AP +0.5, ML ±3.0, DV −6.5 relative to the bregma and dural surface, according to the atlas of Paxinos and Watson (1996). Probes were perfused with a CMA-102 pump (CMA/Microdialysis AB, Stockholm, Sweden) at a rate of 2 μl/min with artificial cerebrospinal fluid (aCSF) containing (mM): 120 NaCl, 2.8 KCl, 25 $NaHCO_3$, 1.2 $CaCl_2$, and 1.0 $MgCl_2$, at pH 7.2, adjusted by bubbling of the solution with 5% CO_2. Sampling commenced at least 90 minutes after probe implantation and the dialysate samples stored at −20°C until analyzed.

HPLC detection of amino acids

The concentrations of taurine, glutamate, threonine and glycine were measured using HPLC with fluorescent detection after precolumn derivatization with *o*-phtaldialdehyde (OPA) (Kendrick et al., 1996) using the system of Shimadzu Scientific Instruments (Kyoto, Japan). Derivatization was performed in a SIL-10AD autoinjector with the OPA reagent (OPA, 0.4 g/l, 0.25% mercaptopropionic acid and 2% methanol in 0.29 M borate buffer, pH 10.4). The OPA reagent (26 μl) was added to the sample (56 μl), mixed, incubated for 2 min at 4°C, and then injected into the column. The amino acid derivatives were separated using a C18-HC column, ODS 2.5 μm packing, 4.6 × 250 mm (Waters, UK) equipped with a precolumn (4 × 6 mm). The flow rate was set at 0.8 ml per minute. The low-pressure gradient mode was used for elution. The mobile phases were 10% methanol and 2% tetrahydrofuran in 0.067 M phosphate buffer, pH 6.8 (A), and 60% methanol and 2% tetrahydrofuran in water (B). The concentration of B during separation was gradually increased from 3% to 100% and then dropped to zero. Fluorescence was measured with an RF-10A detector using excitation and emission wavelengths set at 340 nm and 450 nm, respectively. The concentrations of amino acids in dialysates were calculated using external and internal standards with VPclass5 software.

Experimental design

Zero net flux experiments and basal taurine release. To ascertain the actual extracellular concentration of taurine we applied zero net flux approach (Lönnroth et al., 1987). At the beginning of the experiment three 20-min basal samples were collected. Thereafter, the probes were perfused with aCSF containing randomly 1, 5, 10, 30 and 50 μM taurine. Three samples were collected from each perfusate solution. The administration of the next solution started 20 min after the previous one.

Assays of TTX, calcium, GES and SITS dependency. In these experiments the animals were divided into five groups: control, TTX, Ca^{2+}-free, GES and SITS groups. The control group received no treatment and ten 20-min samples were collected during the course of the experiment. In the TTX group, three basal samples were collected at the beginning of the experiment, followed by perfusion of 10 μM TTX dissolved in aCSF. In the Ca^{2+}-free group, the probes were perfused with Ca^{2+}-free aCSF after basal sample collection (concentrations of other components in aCSF were kept unaltered). The SITS and GES groups were infused with either the anion channel blocker SITS (2 mM in aCSF) or the competitive inhibitor of taurine transport GES (1 mM in aCSF) via the microdialysis probes from the fourth sample onwards.

Data analysis

Zero net flux experiments and basal taurine release. The basal level of taurine in the dialysate was calculated as an average ± S.E.M. from the first three samples collected in each experiment after perfusion with aCSF. The actual concentration and extraction fraction of taurine were then obtained using linear regression analysis from the data at different taurine concentrations in the perfusion fluid (Harris 2003). The difference between the taurine concentration in the perfusate and the dialysate versus the taurine concentration in the perfusate was plotted. Thus, y and x-axes show the gain or loss of taurine during microdialysis and the concentration of taurine perfused, respectively. The slope of the straight line, calculated with Excel software, represents the extraction fraction. The intercept on

the x-axis represents the point where there is no net flux of taurine across the dialysis membrane. At this point, the concentration of taurine, added to the perfusion medium, is equal to the actual extracellular concentration of taurine.

TTX, calcium, GES and SITS dependency. The mean concentration of amino acids in the first three samples was considered as 100%. The changes in amino acid concentrations evoked by treatments are shown as percentages of this baseline value. The data obtained were compared with the corresponding control values from separate experiments. The statistical significance of differences was estimated using the Student's t-test. The level of significance was set to 0.05.

Results

Basal taurine release and actual extracellular taurine concentration

The results from the zero net flux experiments at different taurine concentrations showed good linearity (Fig. 1). The actual extracellular concentration of taurine in the rat striatum was estimated to be $25.2 \pm 5.1\,\mu$M (Table 1). The basal taurine release into the dialysates was $2.70 \pm 0.28\,\mu$M and the extraction fraction (*in vivo* recovery) was 0.079.

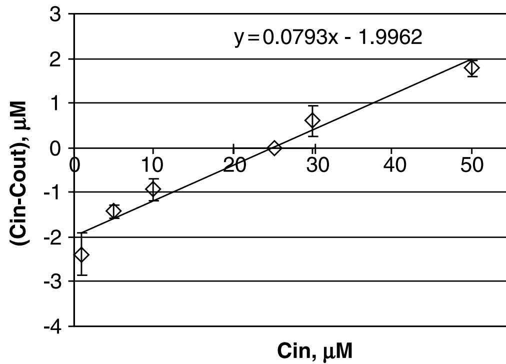


Fig. 1. Zero net flux experiments. The difference between the taurine concentrations in perfusate (C_{in}) and dialysate (C_{out}) is plotted against the concentration of taurine in perfusate. Number of experiments 12. Data are presented as mean values ± S.E.M.

Table 1. Estimated parameters of zero net flux experiment

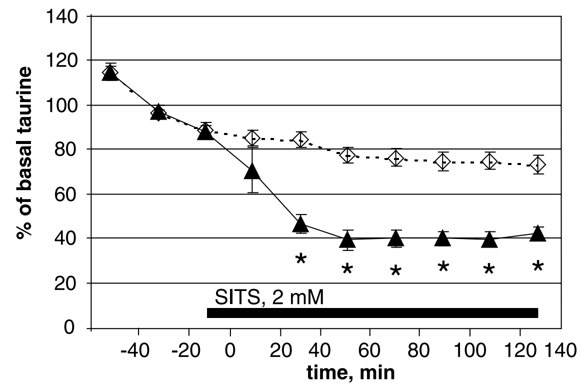
Parameter	Value
Basal taurine release (perfusate concentration)	$2.70 \pm 0.28\,\mu$M
Slope (*in vivo* recovery, extraction fraction)	0.079
r^2 value of linear regression	0.963
Intercept with x-axis (extracellular concentration of taurine)	$25.2 \pm 5.1\,\mu$M

The data were estimated from 12 independent experiments. Mean values ± S.E.M.

Regulation of basal taurine release

The basal taurine release in these experiments ($2.65 \pm 0.20\,\mu$M, n = 22) did not differ significantly from the value obtained with the zero net flux experiments. During the course of sampling the release of taurine gradually decreased. The basal release of glutamate, threonine and glycine was 0.24 ± 0.03, 2.31 ± 0.15 and $1.31 \pm 0.08\,\mu$M, respectively (n = 22). The release of threonine and glycine was stable during the whole time course of the study, whereas the level of glutamate decreased.

Long-term application of the anion channel blocker SITS (2 mM, 140 min) significantly reduced the interstitial taurine concentrations (Fig. 2A). The maximal effect was discernible one hour after commencement of SITS perfusion, constituting a $39.1 \pm 4.6\%$ decrease. The



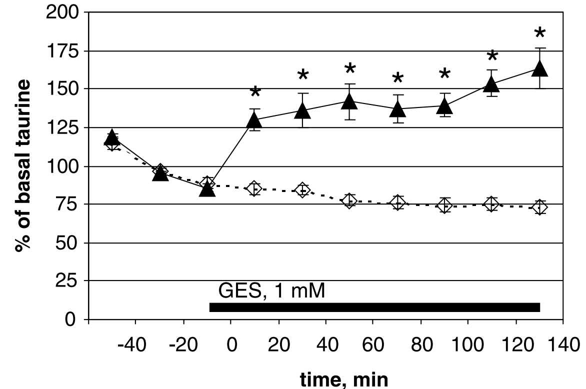


Fig. 2. Extracellular taurine concentrations under perfusion with 2 mM SITS (**A**) and 1 mM GES (**B**) (solid lines, n = 4). $^{*}P < 0.05$, compared to control values (dashed lines, n = 6). The solid bar denotes the period of SITS (**A**) or GES (**B**) perfusion. Data are presented as percentages ± S.E.M. from the baseline

Table 2. Effect of perfusion with 2 mM SITS and 1 mM GES on the extracellular concentrations of glutamate, glycine and threonine

Amino acid	Control % of the baseline	2 mM SITS % of baseline	1 mM GES % of baseline
Glutamate	82.8 ± 1.6	not estimated	80.7 ± 1.8
Glycine	101.6 ± 1.6	98.2 ± 1.8	97.2 ± 5.0
Threonine	105.9 ± 1.9	95.1 ± 2.1	101.7 ± 2.0

Data from 4–6 independent experiments, representing the mean values ± S.E.M. of the fourth to tenth samples (from 20 to 140 min). The baseline is the mean values of the three first samples before application of the effectors. In control experiments the rat brain was perfused with plain aCSF

concentrations of threonine and glycine were not affected by SITS (Table 2). Unfortunately, detection of glutamate was not possible due to the high degree of contamination by SITS at the beginning of the chromatograms.

The extracellular concentration of taurine was elevated by perfusion of the competitive taurine transport inhibitor GES (1 mM, 140 min; Fig. 2B). Taurine release was already significantly enhanced in the first sample after GES application. The maximal release (163.2 ± 13.0) was detected after 140 min. At the same time glutamate, threonine and glycine were not affected (Table 2).

Application of TTX (10 μM, 140 min) led to a gradual increase in the interstitial taurine concentration in the striatum of anesthetized rats (maximal increase was 110.0 ± 3.4% from baseline). The effect became statistically significant after 40 minutes of TTX perfusion (Fig. 3A). Glutamate was also released in response to TTX. The changes in glutamate were of the same magnitude as those in taurine (Fig. 3B). Perfusion with TTX did not influence the interstitial concentrations of threonine and glycine (Fig. 3C, D).

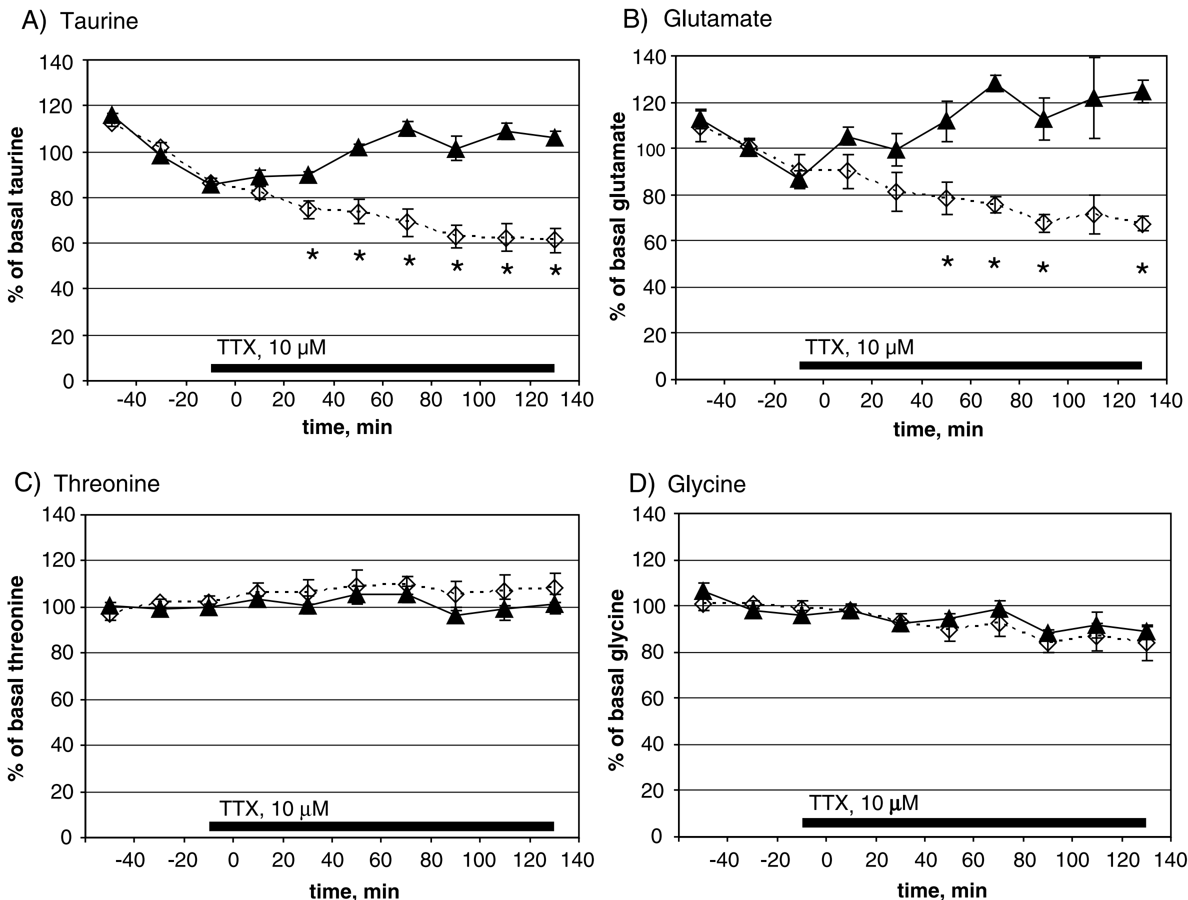


Fig. 3. Effect of perfusion with 10 μM TTX on the taurine (**A**), glutamate (**B**), threonine (**C**) and glycine (**D**) concentrations (solid lines, n = 4). $^{*}P < 0.05$, compared to control values (dashed lines, n = 4). The solid bar denotes the period of TTX perfusion. Data are presented as percentages ± S.E.M. from the baseline

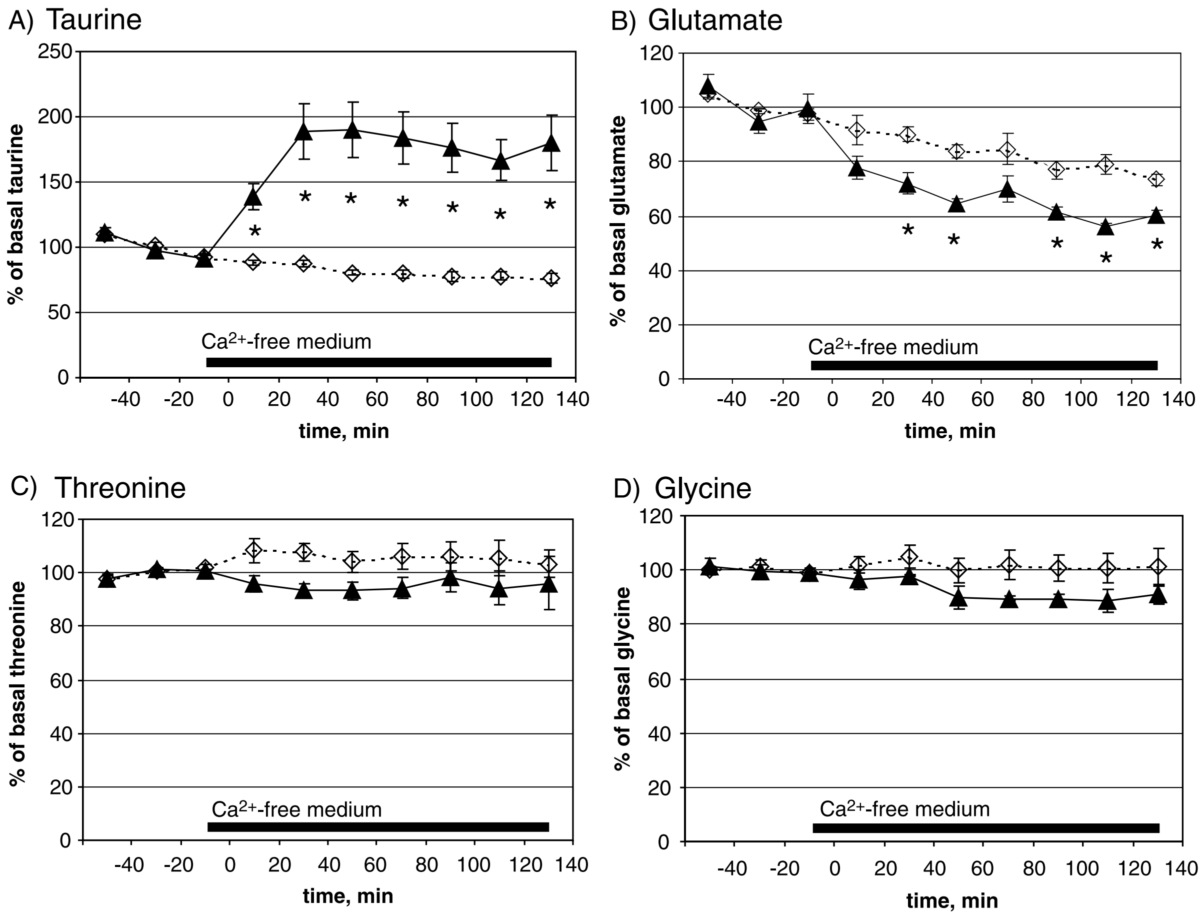


Fig. 4. Effect of perfusion with Ca^{2+}-free medium on the taurine (**A**), glutamate (**B**), threonine (**C**) and glycine (**D**) concentrations (solid lines, n = 4). $^{*}P < 0.05$, compared to control values (dashed lines, n = 6). The solid bar denotes the period of perfusion with Ca^{2+}-free medium. Data are presented as percentages ± S.E.M. from the baseline

Perfusion with Ca^{2+}-free aCSF during 140 min evoked a profound and stable increase in the taurine concentration in the dialysate, that was maximal after one hour (189.6 ± 21.2% from baseline, Fig. 4A). The effect was already statistically significant after 20 min of Ca^{2+}-free aCSF administration. Omission of Ca^{2+} led to a small but statistically significant decrease in the interstitial glutamate concentration, while the concentrations of threonine and glycine were not affected (Fig. 4B–D).

Discussion

Taurine abounds in the brain of many mammalian species at concentrations comparable to those of glutamate (Oja and Kontro, 1983; Huxtable, 1989), but its extracellular concentration in the rat striatum was now shown to be more than ten times higher that of glutamate (Miele et al., 1996; Lai et al., 2000). The present extraction fraction of taurine (*in vivo* recovery) does not markedly differ from the *in vitro* extraction fraction previously estimated in our laboratory (Hilgier et al., 2003). The fraction characterizes the penetration rate of taurine through the microdialysis membrane.

Taurine release evoked by ischemia, (Phillis et al., 1997), high potassium concentrations (Saransaari and Oja, 1998) and hypo-osmotic stimuli (Pasantes-Morales et al., 1990; Estevez et al., 1999) is partially mediated by volume-sensitive anion channels. SITS was used to evaluate whether this pathway is also active under resting conditions. This substance is a potent anion channel blocker and a weak inhibitor of the glutamate transporter. One mM SITS does not change the extracellular glutamate concentration and acts only on anion channels (Tauskela et al., 2003). We infer from the decrease in the extracellular concentration of taurine after SITS

infusion that about one half of taurine exits through the volume-sensitive chloride channels.

The taurine transporter TauT mediates taurine influx into cells. It belongs to the family of sodium- and chloride-dependent neurotransmitter transporters (Tappaz, 2004). The uptake is driven by the membrane gradient of Na^+ and regulated by the Ca^{2+}-calmodulin, protein kinase C and cAMP/protein kinase A pathways. The uptake of taurine is well characterized *in vitro* (Huxtable, 1989). Using the taurine transport inhibitor GES we now also demonstrated active uptake of taurine under resting conditions *in vivo*.

Two classical criteria for neurotransmitter release are the opening of fast sodium channels and Ca^{2+} dependency. Since the extracellular concentrations of both glutamate and taurine were increased by TTX application, but that of threonine not, the present TTX effect seems to be a characteristic of neuroactive amino acids. However, previous results on TTX effects on taurine and glutamate release are discrepant. For instance, the basal release of taurine in the substantia nigra *in vivo* and from brain slices *in vitro* has been slightly reduced or unaffected by inhibition of voltage-gated Na^+ channels by TTX (Biggs et al., 1995; Bianchi et al., 1999). In several studies no changes or a minor decrease in the glutamate concentration have been seen in response to inhibition of the voltage-gated sodium channels (for review see Timmerman and Westerink, 1997). However, in the studies specifically conducted on the striatum, extracellular glutamate has increased (Keefe et al., 1993; Morari et al., 1996). This phenomenon may reflect TTX-mediated disinhibition of cortical glutamatergic terminals. The firing of cortical and thalamic motoneurons is controlled by the striato-thalamo-cortical loop (Parent and Hazrati, 1995a, b). Under resting conditions the striatal efferents are silent, which leaves thalamic neurons suppressed. The dopaminergic striatonigral projections, which are tonically active, balance the activity of the two branches of the loop. Intrastriatal application of TTX attenuates dopaminergic tone and leads to disinhibition and subsequent release of glutamate from the cortical inputs.

Taurine was now released upon TTX application in a similar manner and in the same order of magnitude as glutamate. Taurine is thus either released from the same site (cortical inputs) or glutamate induces taurine release from other sources. It has previously been reported that decortication reduces the striatal levels of glutamate, but leaves the taurine concentrations unchanged (Butcher and Hamberger, 1987). The TTX-induced taurine elevation is hence likely to occur secondarily after the glutamate release. Although taurine may be released by a non-synaptic mechanism, the release is strongly coupled to changes in neuronal activity.

Due to methodological problems it is difficult to prove the Ca^{2+} dependency of the release measured by microdialysis. The basal glutamate release has been found to be unchanged or only slightly decreased after omission of Ca^{2+} (for review see Timmerman and Westerink, 1997). Here, a minor but statistically significant decrease in extracellular glutamate was likewise discernible in Ca^{2+}-free medium. The concentration of threonine was stable, indicating that Ca^{2+} omission does not evoke changes in non-neuroactive amino acids. In contrast, taurine release was enhanced in the absence of Ca^{2+}. Enhanced taurine release in the presence of Ca^{2+} chelators has already been seen in brain slices (Korpi and Oja, 1984; Oja et al., 1985; Saransaari and Oja, 1992), though the mechanism of this enhancement is not known. Interestingly, the omission of Ca^{2+} has no effect on taurine release in the supraoptic nucleus of the hypothalamus, where taurine is localized mainly in glia (Decavel and Hatton, 1995; Hussy et al., 2000). Since the localization of taurine in the striatum is predominantly neuronal (Storm-Mathisen and Ottersen, 1986; Madsen et al., 1987; Della Corte et al., 1990), enhanced taurine release in Ca^{2+}-free solution may be a phenomenon specific for neurons, but not for glia.

The taurine actions in the striatum are partially mediated by strychnine-sensitive glycine receptors (Chepkova et al., 2002). Glycine is a major inhibitory neurotransmitter in the spinal cord and brain stem. Recent studies have demonstrated the expression of functional glycine receptors in the forebrain neurons, but their physiological role is unknown (Sergeeva and Haas, 2001). Since glycinergic transmission in the forebrain has not yet been shown, these glycine receptors may be the target of either glycine or taurine (Flint et al., 1998; Hussy et al., 2000). In the rat striatum, strychnine-sensitive glycine receptors are present on cholinergic interneurons (Darstein et al., 2000), and taurine, together with glycine, is their highly potent agonist (Sergeeva and Haas, 2001). In our study, neither TTX nor Ca^{2+} omission had any effect on the extracellular concentration of glycine, signifying that taurine is rather the endogenous agonist of striatal glycine receptors.

Conclusions

We report that under resting conditions extracellular taurine concentrations are high and undergo dynamic

regulation by volume-regulated Cl^- channels and taurine transporter. In contrast to glutamate, Ca^{2+} depletion enhances basal taurine release suggesting that synaptic exocytosis is not involved. From the similar alterations in taurine and glutamate after TTX application we conclude that taurine release is related to neural activity but by means of some non-synaptic mechanism. Since neuronal activation alters ion gradients across cell membranes, taurine is likely to be released via osmotically induced pathways.

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Authors' address: Svetlana Molchanova, Brain Research Center, Medical School, 33014 University of Tampere, Finland,
Fax: +358 3 215 6170, E-mail: svetlana.molchanova@uta.fi

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Interstitial Concentrations of Amino Acids in the Rat Striatum During Global Forebrain Ischemia and Potassium-Evoked Spreading Depression

Svetlana Molchanova,[1,4,5] Peeter Kööbi,[2] Simo S. Oja,[1,3] and Pirjo Saransaari[1]

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The early detection and appropriate treatment of brain ischemia is of paramount importance. The interstitial concentrations of neurotransmitter amino acids are often used as an index of neuronal injury. However, monitoring of non-neurotransmitter amino acids may be equally important. We have studied the behavior of 10 amino acids during K^+-induced spreading depression (application of 70 mM KCl during 40 min) and global forebrain ischemia (two-vessel occlusion with hypotension during 20 min). The concentrations of glutamate, aspartate, taurine, GABA, glycine, and alanine, measured in the rat striatum by microdialysis, increased during both ischemia and spreading depression, whereas glutamine concentrations decreased in both cases. Only ischemia, but not spreading depression, led to enhanced release of serine, threonine, and asparagine. We thus conclude that an elevation in the interstitial concentrations of non-neurotransmitter amino acids is specific to deep ischemic injury to nervous tissue. We propose the monitoring of serine, asparagine, and threonine, together with excitatory amino acids, as an index of the degree of ischemic brain injury.

KEY WORDS: Amino acids; ischemia; microdialysis; spreading depression; striatum.

INTRODUCTION

Ischemic brain damage is frequently seen in patients resuscitated from cardiac arrest or circulatory failure during surgical interventions. Because brain cells actually die rapidly upon ischemia, the early detection and appropriate treatment of ischemic injury is of paramount importance. Among other methods, special attention is paid to microdialysis to monitor and characterize the state of nervous tissue under cardiac or brain vessel surgery (for review, see 1–3). A decrease in the oxygen and glucose supply initiates reactions that lead to cell death by apoptotic or necrotic pathways (4). Excitatory neurotransmitter glutamate is claimed to be responsible and the main inherent indicator of ischemic cell injury (5). Extracellular glutamate has successfully predicted the outcome after partial circulatory failure in humans upon surgery (6,7). Other amino acids may also be important in monitoring the development of brain injury and predicting prognosis. The effective modern techniques allow us to measure simultaneously a number of amino acids as routine monitoring (8,9).

[1] Brain Research Center, Medical School, University of Tampere, Tampere, Finland.
[2] Department of Pharmacological Sciences, University of Tampere, Tampere, Finland.
[3] Department of Clinical Physiology, Tampere University Hospital, Tampere, Finland.
[4] I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry, RAS, St. Petersburg, Russia.
[5] Address reprint requests to: Svetlana Molchanova, Brain Research Center, Medical School, FIN-33014 University of Tampere, Finland. Tel: +358 3 215 6715; Fax: +358 3 215 6170; E-mail: svetlana.molchanova@uta.fi

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The interstitial concentrations of amino acids could be used as an index of the *degree* of neuronal injury. Ischemia causes release of amino acids by a number of mechanisms, which are more or less dependent on the degree of injury and specific for the amino acid (10). During the first 1–2 min the initial efflux of amino acids in ischemia induced by membrane depolarization is Ca^{2+}-dependent, comprising neurotransmitter amino acids. The subsequent efflux comprises nonvesicular Ca^{2+}-independent release, mediated in part by Na^{+}-dependent amino acid transporters in the plasma membrane operating in a reversed mode, and by the swelling-induced opening of chloride channels. The swelling cells attempt to restore their normal volume by extruding osmotically active solutes. The amino acids released during the regulatory volume decrease include aspartate, taurine, glutamate, GABA, and glycine (11). Finally, disruption of the membrane integrity provides general exodus of amino acids during the last steps of ischemic injury. Neurotransmitter amino acids such as glutamate are released in ischemia by means of all the above pathways (12), but only little information is available concerning non-neurotransmitter amino acids, which may best reflect the last mode of release and hence best indicate the degree of ischemic injury.

The initial ischemic injury to nervous tissue is characterized by inhibition of oxidative phosphorylation, decreased pH and ATP, increased cellular Na^{+}, and membrane depolarization. Increased extracellular potassium and subsequent depolarization in ischemia trigger generation of spreading depression (SD) waves, which propagate along the brain tissue (13). During SD the EEG is depressed, DC potentials undergo substantial shifts, and water moves from the extracellular to intracellular compartments (14,15). SD, evoked by electrical stimuli or by application of excessive concentrations of KCl, is not harmful to normal nervous cells (16) but initiates large amino acid release (17). Of the four aforementioned modes of ischemic release of amino acids, the first three take place also during SD due to depolarization and cell swelling caused by disbalance in K^{+} and Cl^{-} (18). However, because neurons are viable during SD, the general amino acid exit consequent upon membrane disruption does not occur.

We now describe the behavior of 10 amino acids, both neuroactive and non-neuroactive, during ischemia and SD. Global ischemia associated with severe circulatory failure was mimicked by the rat model of global forebrain ischemia, established by two-vessel occlusion with hypotension during 20 min. Long-lasting K^{+}-evoked spreading depression (40 min) was used to model the ischemia-like amino acid release under circumstances that cause no neuronal damage.

EXPERIMENTAL PROCEDURE

Animals. The studies were carried out on anesthetized Sprague-Dawley male adult rats weighing 250–300 g. The experimental procedures were in accordance with the European Community Directive for the ethical use of experimental animals. All efforts were made to minimize both suffering and number of animals used.

Microdialysis Surgery. The implantation of microdialysis probes was done on the day of experiments under halothane narcosis. The animals were first anesthetized with 4% halothane in air and placed in a stereotaxic frame (David Kopf Instruments, Düsseldorf, Germany). The concentration of halothane was then reduced to 0.8%–1.2% and body temperature kept at 37.5°C with a heating pad. A microdialysis probe of concentric design (4-mm length, 0.5-mm o.d.) was implanted in the striatum. In the experiments with K^{+} stimulation, two probes were used at the same time, the control and stimulated sides being chosen randomly. The stereotaxic coordinates were as follows: AP +0.5, ML ±3, DV −6.5 relative to the bregma and dural surface, according to the atlas of Paxinos and Watson (19). The probes were perfused with a CMA-102 pump (CMA/Microdialysis AB, Stockholm, Sweden) at a rate of 2 μl/min with artificial cerebrospinal fluid (aCSF) containing (mM) 120 NaCl, 2.8 KCl, 25 $NaHCO_3$, 1.2 $CaCl_2$, and 1.0 $MgCl_2$, at pH 7.2, adjusted by bubbling of the solution with 5% CO_2.

To establish SD conditions (20), the concentration of KCl in aCSF was increased to 70 mM with an appropriate decrease in NaCl (52.8 mM), whereas the concentrations of other salts were not changed. Sampling was started at least 2 h after probe implantation, and the samples of dialysates were stored at −20°C until analyzed.

Ischemic Surgery. After implantation, the probe was fixed on the skull with screws and dental cement and the animal placed in supine position. The carotid arteries were exposed and a catheter (PE 50, Clay-Adams, Parsippany, NJ, USA) was inserted into the femoral artery in order to measure the mean arterial blood pressure (MABP) and for withdrawal of blood. Forebrain ischemia was induced by bilateral carotid occlusion with controlled hypotension. A mean pressure of 50 mm Hg was achieved by withdrawal of blood into a heparinized syringe. Immediately after establishing hypotension, both carotid arteries were clamped with clips. After the 20-min ischemic period, the clips were removed and MABP increased close to the basal level (Table I).

HPLC Detection of Amino Acids. The concentrations of aspartate, glutamate, asparagine, serine, glutamine, glycine, threonine, alanine, taurine, and GABA were measured by HPLC with fluorescent detection after precolumn derivatization with *o*-phtaldialdehyde (OPA) (9) using the system of Shimadzu Scientific Instruments (Kyoto, Japan). Derivatization was performed in a SIL-10AD autoinjector with the OPA reagent (OPA, 0.4 g/L, 0.25% mercaptopropionic acid and 2% methanol in 0.29 M borate acid, pH 10.4). The OPA reagent (26 μl) was added to the sample (56 μl), mixed, incubated for 2 min at 4°C, and then injected into the column. The amino acid derivatives were

Table I. Mean Arterial Blood Pressure (MABP) and Body Temperature During Ischemia

Parameter	Basal	Ischemia	Reperfusion
MABP	96.3 ± 6.1	55.7 ± 1.8	107.7 ± 8.9
Body temperature	37.5 ± 0.1	37.6 ± 0.1	37.4 ± 0.1

Note: Mean values (mm Hg) with SEM.

separated using a C18-HC column, ODS 2.5 μm packing, 4.6 × 250 mm (Waters, Elstree, Hertsfordshire, UK) equipped with a pre-column (4 × 6 mm). The flow rate was set at 0.8 ml per min and the low-pressure gradient mode used for elution. The mobile phases were 10% methanol and 2% tetrahydrofuran in 0.067 M phosphate buffer, pH 6.8 (A), and 60% methanol and 2% tetrahydrofuran in water (B). The concentration of B during separation was gradually increased from 3% to 100% and then dropped to zero. Fluorescence was measured with an RF-10A detector using excitation and emission wavelengths set at 340 nm and 450 nm, respectively. The concentrations of amino acids in dialysate were calculated using external and internal standards with VPclass5 software.

Data Analysis. In the experiments, the mean concentration of an amino acid in the first three samples was defined as 100%. The changes in the amino acid concentrations evoked by the treatments are then shown as percentages of this baseline. The statistical significance of differences was estimated with Student's *t* test, in which the level of significance was set at $P < 0.05$.

RESULTS

Basal Amino Acid Levels

The basal concentrations of amino acids in the dialysates are given in Table II. Glutamine was the most dominant amino acid, whereas the levels of GABA, aspartate, and asparagine were rather low.

Spreading Depression

Neurotransmitter and Neuromodulatory Amino Acids

Perfusion with aCSF containing 70 mM KCl induced significant release of glutamate and aspartate, sixfold and fourfold, respectively, during the first 20 min (Figs. 1A and 1B). The elevated concentrations were maintained during SD and immediately decreased after the cessation of high-KCl perfusion. Other neurotransmitter and neuromodulatory amino acids were also released during SD (Figs. 1C and 1D and Fig. 2F). GABA increased 12-fold, taurine 8-fold, and glycine 1.5-fold. The GABA release reached the maximum in the first part of SD and returned to baseline after the onset of control conditions. Other two amino acids maintained high concentrations for a longer time, and the level of taurine was still more than twofold higher than the basal level 1 h after SD.

Table II. Mean Basal Concentrations of Amino Acids in the Dialysates

Amino acid	Concentration	Amino acid	Concentration
Aspartate	0.14 ± 0.06	Glycine	0.90 ± 0.26
Glutamate	1.79 ± 0.29	Threonine	1.66 ± 0.27
Asparagine	0.23 ± 0.07	Alanine	1.79 ± 0.40
Serine	2.75 ± 0.53	Taurine	2.26 ± 0.60
Glutamine	19.21 ± 4.49	GABA	0.13 ± 0.04

Note: The concentrations with SEM are given as μM.

Other Amino Acids

SD evoked a 1.5-fold release of alanine (Fig. 2A); the concentrations in the dialysate mimicked the changes in glycine. The concentrations of serine and threonine were not markedly changed during perfusion with high KCl (Figs. 2B and 2C). Both amino acids were slightly decreased after the KCl administration period. However, a trend to decrease was already discernible during the whole experiment. The concentration of asparagine was not changed under SD conditions (Fig. 2D). At the same time, glutamine dramatically declined to one fifth during prolonged SD and recovered only after 1 h of perfusion with control aCSF (Fig. 2E).

Ischemia

Neurotransmitter and Neuromodulatory Amino Acids

During ischemia, the concentrations of excitatory amino acids increased dramatically. The maximum concentrations were observed at 20 min after the onset of ischemia. A 7-fold increase in glutamate and a 20-fold increase in aspartate were reached (Figs. 3A and 3B). Both amino acids returned to the basal levels during reperfusion. Glutamate decreased immediately upon onset of reperfusion, whereas aspartate approached the control values after 20 min. Ischemia also induced profound increases in the interstitial amounts of GABA (maximally 36-fold), taurine (30-fold), and glycine (5-fold) at 20 min after the onset of ischemia (Figs. 3C and 3D and Fig. 4F). GABA and glycine decreased fairly rapidly during reperfusion, but the taurine concentration still remained at the ischemic level in the first reperfusion sample. It approached the basal level only one hour after the end of ischemia.

Other Amino Acids

Ischemia elevated the extracellular amounts of alanine, serine, threonine, and asparagine in the same manner as those of neuroactive amino acids (Figs. 4A–4D), but the magnitudes of changes were less, varying from twofold (threonine) to fourfold (alanine), being nonetheless statistically significant. All concentrations returned to the basal levels during the reperfusion period. The concentration of glutamine in dialysates was not altered during ischemia, but was reduced to one half of the basal level within the first 20 min of reperfusion (Fig. 4E).

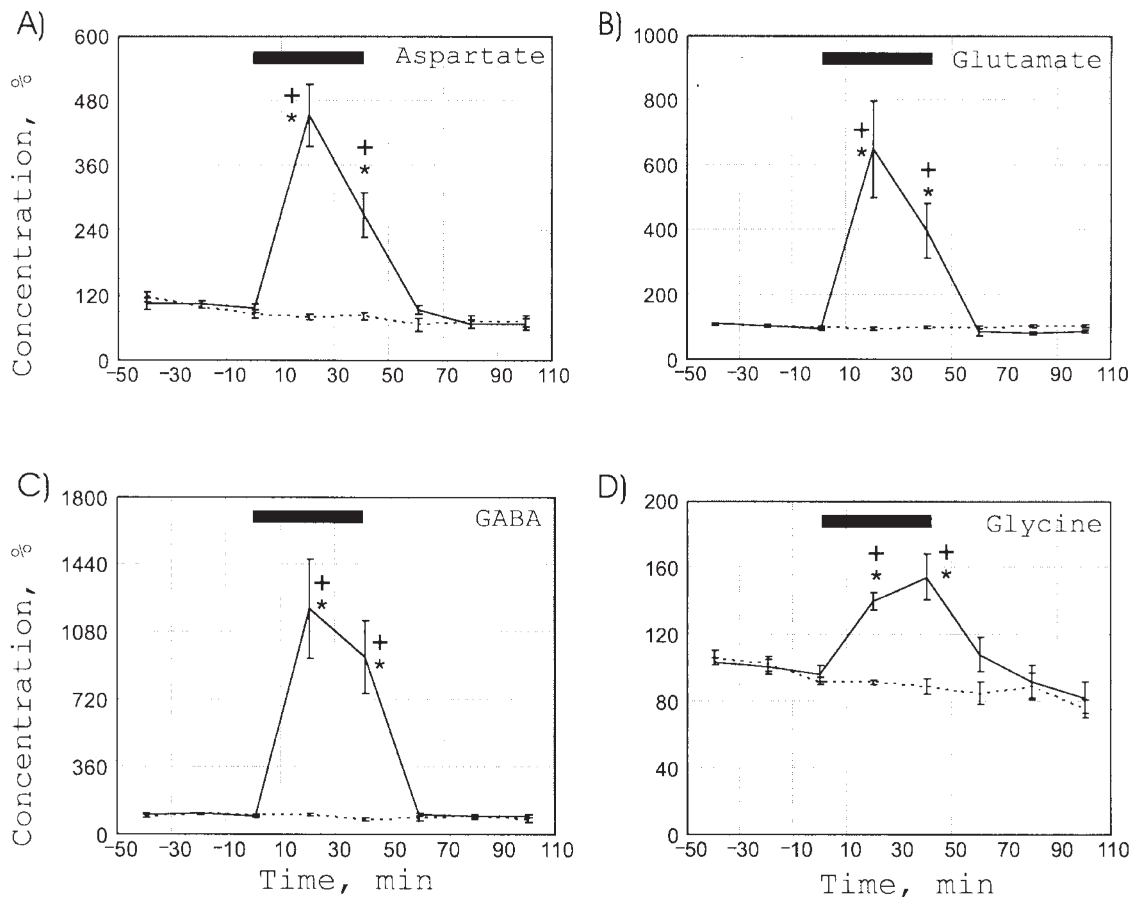

Fig. 1. Percentage changes in the interstitial concentrations of (A) aspartate, (B) glutamate, (C) GABA, and (D) glycine during spreading depression. Solid line, SD; dotted line, control; solid bar, 70 mM KCl, 40 min; $^{*}P < 0.05$ compared to baseline and $^{+}P < 0.05$ compared to control.

DISCUSSION

Neurotransmitter and Neuromodulatory Amino Acids

Ischemia evokes substantial release of aspartate and glutamate, which is rapidly attenuated upon commencement of reperfusion (21,22). Spreading depression causes similar effects but of lesser magnitude (12). The current data tally well with the earlier studies mentioned. GABA and glycine are likewise substantially released in ischemia (22,23) and SD (12). Our results also confirm these previous observations, but at variance with them we failed to detect any significant prolonged stimulation of glycine release. In addition to the well-established action of glycine as a co-agonist of NMDA receptors, it seems also to be inhibitory in the striatum due to the presence of strychnine-sensitive glycine receptors in this structure (24,25). The possible dual function of glycine in the striatum may explain the difference between our data and the results published earlier. The sulfur-containing amino acid taurine, an inhibitory neuromodulator (26), plays an important role in regulatory volume adjustments of neurons to hypo-osmotic stimuli (27). Taurine is released during ischemia and SD and still remains elevated during reperfusion (17,23). Such delayed responses are characteristic of this amino acid both *in vitro* (28) and *in vivo* (20). Taurine is apparently neuroprotective, and the increased extracellular contents of this amino acid may reflect compensatory mechanisms activated in response to injury. In particular, the late peak release of taurine and the slow recovery after ischemic and depolarizing conditions reflect this function. The behavior of amino acids, which serve as neurotransmitters and neuromodulators in the nervous system, is quite predictable. They are all released in response to both ischemia and SD. Under depolarizing conditions, the release is mainly Ca^{2+}-dependent, and this is also the case in ischemia during the first minutes. Thereafter, due to the ion disbalance, the release is mainly mediated by membrane transport systems, operating in the reverse mode, and cell swelling (10).

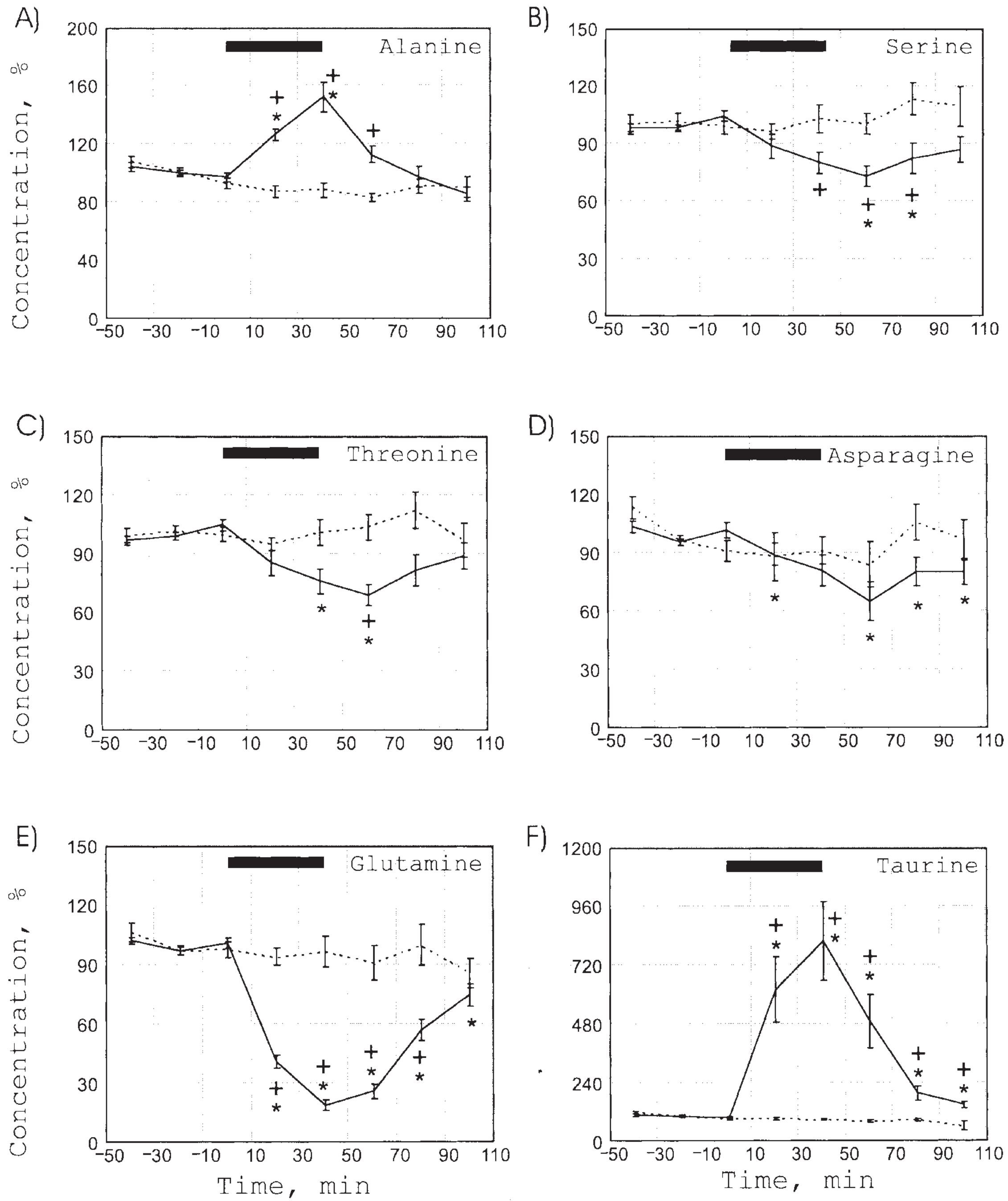

Fig. 2. Percentage changes in the interstitial concentrations of (A) alanine, (B) serine, (C) threonine, (D) asparagine, (E) glutamine, and (F) taurine during spreading depression. Solid line, SD; dotted line, control; solid bar, 70 mM KCl, 40 min; $*P < 0.05$ compared to baseline and $^{+}P < 0.05$ compared to control.

Other Amino Acids

Both ischemia and SD reduce the release of glutamine, the metabolic precursor of both glutamate and GABA (17,29), apparently reflecting the increased glutamine use for neurotransmitter synthesis and activation of phosphate-activated glutaminase (30). Unfortunately, no earlier literature on asparagine during ischemia (metabolic precursor of aspartate) could explain its behavior under ischemic conditions and spreading depression.

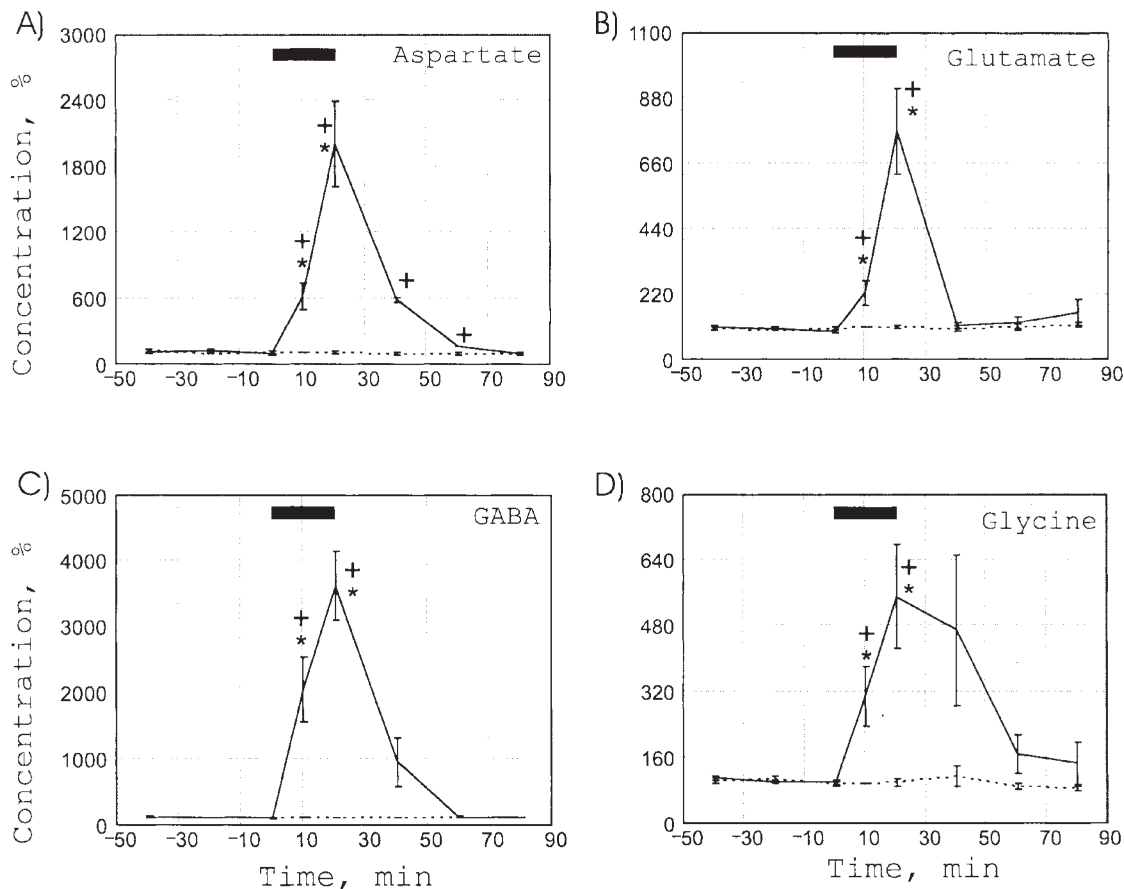

Fig. 3. Percentage changes in the interstitial concentrations of (A) aspartate, (B) glutamate, (C) GABA, and (D) glycine during ischemia. Solid line, ischemia; dotted line, control; solid bar, ischemia period, 20 min; $^*P < 0.05$ compared to baseline and $^+P < 0.05$ compared to control.

Studies regarding the behavior of serine, alanine, and threonine under ischemic conditions are few in number. Alanine is used as a non-neuroactive control when monitoring amino acid concentrations during ischemia (29,31). The elevation in the interstitial concentrations of alanine in response to ischemia is often explained as resulting from the shrinkage of the extracellular space attributable to astrocytic swelling. However SD, even of short duration, causes significant release of this amino acid (12). It has recently been shown that alanine serves as a donor of nitrogen for the *de novo* synthesis of glutamate in astroglia (32). Alanine forms together with lactate a shuttle between neurons and astrocytes, which transports the amino group from neurons to glia. In astrocytes, alanine is converted to lactate and the nitrogen is incorporated in the amide group of glutamine. Lactate moves in the opposite direction, returning the carbon skeleton to the neurons. In addition to the glutamate–glutamine cycle, alanine and lactate thus form an additional cycle responsible for the accumulation of glutamate in glutamatergic neurons. Moreover, alanine is also involved in GABA synthesis. The elevation of interstitial alanine during SD can thus occur because of intensive glutamate synthesis *de novo* and the alanine–lactate exchange. This shuttle requires strong compartmentalization of alanine and the presence of high-affinity alanine transporters. In this case, depolarization in ischemia may perturbate the transport of alanine from neurons to glia. We thus suggest that alanine can be released in response to any stimulus, which enhances neurotransmission. The increased intracellular concentration of alanine can signify an intensive *de novo* synthesis of glutamate and GABA.

The conditionally essential amino acid L-serine is of paramount importance for the metabolism in the nervous system (33). It functions as the predominant source of one-carbon units for the *de novo* synthesis of purine nucleotides. Additionally, the metabolic pathway of serine provides precursors for the synthesis of other amino acids such as taurine, glycine and D-serine. Serine is shown to be elevated in ischemia in animal models (31) and humans (34). Another non-neuroactive amino acid, threonine, is

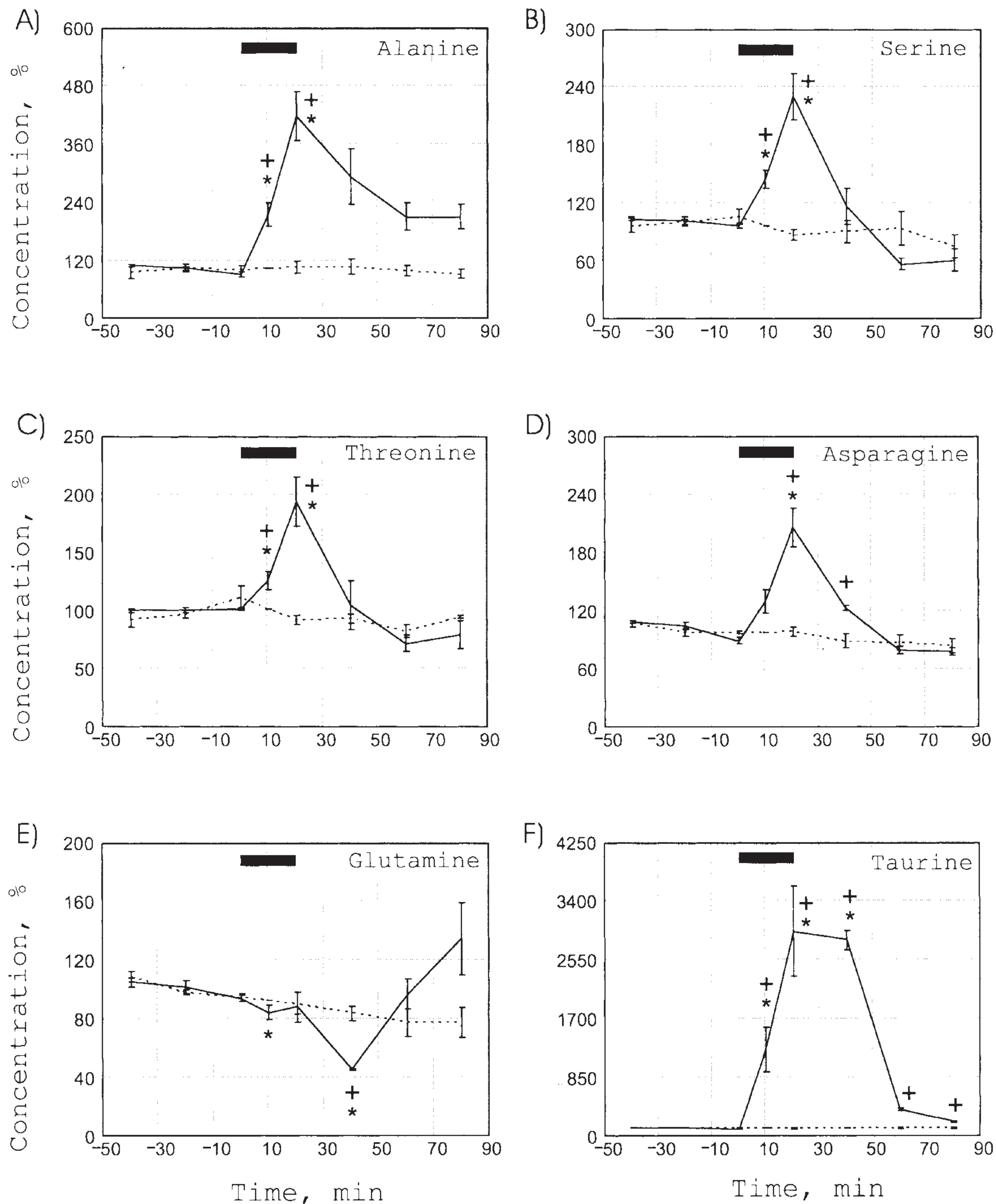

Fig. 4. Percentage changes in the interstitial concentrations of (A) alanine, (B) serine, (C) threonine, (D) asparagine, (E) glutamine, and (F) taurine during ischemia. Solid line, ischemia; dotted line, control; solid bar, ischemia period, 20 min; $^{*}P < 0.05$ compared to baseline and $^{+}P < 0.05$ compared to control.

only used in protein synthesis. It is not known whether this compound serves any special function in the central nervous system. Global ischemia leads to a significant release of serine and threonine, but SD did not cause any changes in the interstitial concentrations of these amino acids.

CONCLUSION

We show that the substantial increase in the interstitial concentrations seen during ischemia is not inherent solely to neurotransmitter amino acids. The ischemia-

induced release is not specific to neuroactive compounds and may be characteristic of many small molecules with a high intra- to extracellular concentration gradient. The non-neurotransmitter amino acids asparagine, serine, and threonine are released only in ischemia but not under depolarizing conditions. We thus suggest that the release of non-neurotransmitter amino acids reflects the disruption of membrane integrity, which takes place in ischemic and excitotoxic injuries due to reactive oxygen species formation and Ca^{2+}-dependent activation of the lipid cleavage (35,36). Such membrane damage seems to be specific to ischemic brain injury and could not be evoked by spreading depression. The recent clinical study by Hutchinson and colleagues (6) provides good confirmation of our results. Microdialysis monitoring of cerebral ischemia in patients with head injury and subarachnoid hemorrhage showed a substantial extracellular increase in neuroactive amino acids in all cases. The non-neurotransmitter amino acids were elevated only in one case with poor recovery.

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BRAIN RESEARCH

Research Report

Taurine attenuates D-[^{3}H]aspartate release evoked by depolarization in ischemic corticostriatal slices

Svetlana M. Molchanova[a,c,*], *Simo S. Oja*[b], *Pirjo Saransaari*[a]

[a]*University of Tampere, Medical School, Brain Research Center, Tampere, Finland*
[b]*Tampere University Hospital, The Centre for Laboratory Medicine, Tampere, Finland*
[c]*I.M. Sechenov Institute of Experimental Physiology and Biochemistry, Russian Academy of Sciences, St.-Petersburg, Russia*

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Abbreviations:
KRH, Krebs–Ringer–HEPES buffer
GES, guanidinoethanesulfonate
GABA, γ-aminobutyric acid
NMDA, N-methyl-D-aspartate
THBA, DL-threo-β-hydroxyaspartate
SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid

ABSTRACT

Taurine is thought to be protective in ischemia due to its neuroinhibitory effects. The present aim was to assess the ability of taurine to attenuate glutamate release evoked by ischemia and to determine which component of this release is affected. The release of preloaded D-[^{3}H]aspartate (a non-metabolized analog of glutamate) from superfused murine corticostriatal slices was used as index of glutamate release. Preincubation of corticostriatal slices with 10 mM taurine reduced the D-[^{3}H]aspartate release evoked by either chemical ischemia (0.5 mM NaCN in glucose-free medium) or oxygen–glucose deprivation. The taurine uptake inhibitor guanidinoethanesulfonate (5 mM), the glycine receptor antagonist strychnine (0.1 mM) and the $GABA_A$ receptor antagonist bicuculline (0.1 mM) did not block the taurine effect. To determine which component of ischemia-induced glutamate release is affected by taurine, three pathways of this release were pharmacologically modeled. Unlabeled D-aspartate (0.5 mM) and hypo-osmotic medium (NaCl reduced by 50 mM) evoked D-[^{3}H]aspartate release via homoexchange and hypo-osmotic release pathways, respectively. Taurine did not influence these pathways. However, it suppressed the synaptic release of D-[^{3}H]aspartate evoked by the voltage-gated sodium channel opener veratridine (0.1 mM). Taurine thus reduces glutamate release under ischemic conditions by affecting the depolarization-evoked component.

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1. Introduction

Ischemic injury to nervous tissue involves a number of processes leading to cell death in vulnerable regions of the brain (Lipton, 1999). Energy failure during ischemia induces a massive release of the excitatory neurotransmitter L-glutamate, which is considered to be the key factor in ischemic cell death. Subsequent overactivation of glutamate receptors initiates an increase in intracellular Ca^{2+}, leading to activation of Ca^{2+}-dependent proteases, phospholipases and endonucleases, hence causing damage to macromolecules and cell death (Choi and Rothman, 1990; Arundine and Tymianski, 2004).

Taurine (2-aminoethanesulphonic acid), an amino acid abounding in the central nervous system of mammals (Huxtable, 1989), is thought to act as an inhibitory

* *Corresponding author*. Fax: +358 3 3551 6170.
E-mail address: Svetlana.Molchanova@uta.fi (S.M. Molchanova).

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neuromodulator and osmoregulator (Oja and Saransaari, 1996; Schaffer et al., 2000; El Idrissi and Trenkner, 2004). It has also been shown to regulate calcium storage in cells and to neutralize hypochlorous acid formed during oxidative stress (Foos and Wu, 2002; Schuller-Levis and Park, 2003; Wu et al., 2005). Due to these activities, taurine may serve as a neuroprotector in ischemic cell injury evoked by excessive extracellular glutamate concentrations. Indeed, exogenously applied taurine alleviates neuronal damage evoked by a variety of pathological impacts, including ischemia, epileptic seizures, energy metabolism perturbation and oxidative stress (O'Byrne and Tipton, 2000; Rivas-Arancibia et al., 2001; El-Abhar and Abd El Gawad, 2003; El Idrissi et al., 2003; Kingston et al., 2004; Wang et al., 2005). Taurine also protects neurons from glutamate-induced cell death (Zhao et al., 1999; El Idrissi and Trenkner, 1999; Chen et al., 2001; Foos and Wu, 2002; Louzada et al., 2004; Wu et al., 2005).

When toxic concentrations of glutamate are applied, taurine supports cell survival by lowering the intracellular level of free Ca^{2+} (Zhao et al., 1999; El Idrissi and Trenkner, 1999; Chen et al., 2001; Foos and Wu, 2002; Wu et al., 2005). It reduces the glutamate-evoked Ca^{2+} influx (Wu et al., 2005). This effect is non-specific and independent of the type of Ca^{2+} channel. Taurine also impedes glutamate-induced membrane depolarization (Wu et al., 2005), probably activating an unknown chloride channel and thus hyperpolarizing plasma membranes. Such a conception is supported by evidence that the neuroprotective effect of taurine may be mediated by γ-aminobutyric acid (GABA) receptors (Louzada et al., 2004). Moreover, it is possible that taurine attenuates not only the synaptic effect of glutamate, but also its release. Indeed, taurine modifies the high K^+-induced release of amino acid transmitters, including glutamate, from nerve terminals isolated from the cerebral cortex (Kamisaki et al., 1993; Zheng, 2001). At variance with this, a microdialysis study of substantia nigra has shown amplification of glutamate release due to taurine administration (García Dopico et al., 2004).

The mechanism of the neuroprotective effect of taurine is unclear, and the exact molecular target or activated signal chain remains unknown. A putative specific taurine recognition site in synaptic membranes has been described (Kontro and Oja, 1987a; Wu et al., 1992; Frosini et al., 2003a,b) but not yet thoroughly characterized. Taurine interferes with the functions of GABA and glycine receptors (Malminen and Kontro, 1986; Kontro and Oja, 1987b; Ye et al., 1997; Belluzzi et al., 2004), but the true role of taurine in excitatory and inhibitory neurotransmission in the brain remains ambiguous. The aim of the present study was to assess the ability of taurine to attenuate the glutamate release evoked by ischemia. Preloaded D-[^{3}H] aspartate (a non-metabolized analog of glutamate) in corticostriatal slices was used as the marker of L-glutamate. We also checked whether the possible molecular target of taurine is located extracellularly and whether $GABA_A$ or glycine receptors are involved. Furthermore, we determined which component of ischemic glutamate release is affected.

2. Results

2.1. *Effects of ischemia and taurine on D-[^{3}H]aspartate release*

Chemical ischemia induced by 0.5 mM NaCN in Krebs–Ringer–HEPES buffer (KRH) which contained no glucose significantly enhanced the rate of D-[^{3}H]aspartate efflux, with an increase of about 25-fold between 46 and 50 min of superfusion (Fig. 1A). The fractional efflux rate constants calculated for the period of established chemical ischemia (40–50 min of superfusion) were also significantly, more than 20-fold, elevated when compared to the basal period (Table 1, Figs. 2A, B). Oxygen–glucose deprivation induced by incubating slices in glucose-free KRH and under N_2 atmosphere also evoked D-[^{3}H]aspartate release, but less markedly. Under these conditions, the rate of D-[^{3}H]aspartate release increased 5- to 6-fold at the end of

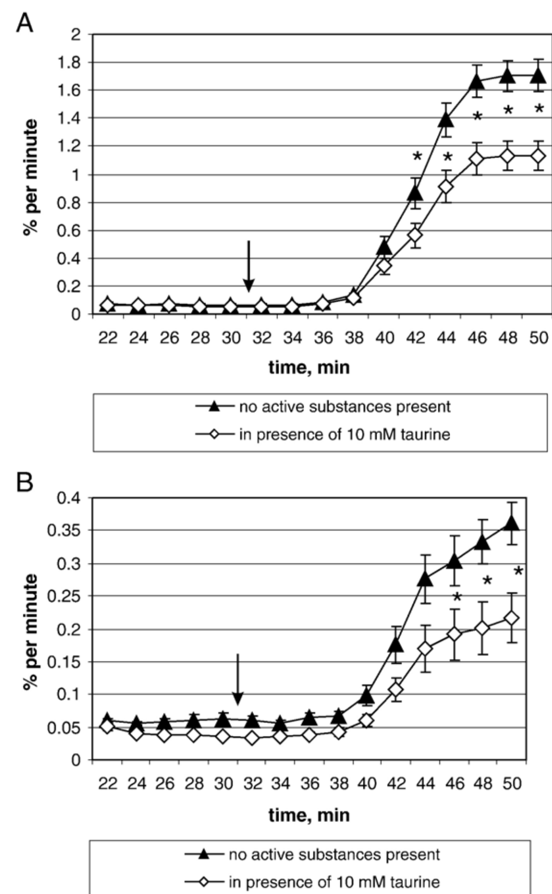

Fig. 1 – Efflux rate of D-[^{3}H]aspartate from corticostriatal slices under ischemic conditions and in the presence of taurine. (A) Chemical ischemia, evoked by 0.5 mM NaCN in glucose-free KRH; (B) oxygen–glucose deprivation. Chemical ischemia and oxygen–glucose deprivation were evoked at the 31st minute of superfusion (shown by arrow). Data are given as mean values ± SEM of 4 to 12 experiments. Taurine was present in incubation medium from the beginning of superfusion. *$P < 0.05$ compared with the efflux rate in the presence of taurine.

Table 1 – Rate constants of D-[3H]aspartate efflux from corticostriatal slices under ischemia and taurine treatment

Experimental conditions	Rate constants of basal release, $k_1 \times 10^3$	Rate constants of evoked release, $k_2 \times 10^3$
Chemical ischemia (12)	0.29 ± 0.03	6.41 ± 0.45[a]
+5 mM taurine (12)	0.28 ± 0.04	7.04 ± 0.55[a]
+10 mM taurine (12)	0.24 ± 0.02	4.26 ± 0.44[a,b]
Oxygen–glucose deprivation (9)	0.26 ± 0.02	1.27 ± 0.14[a]
+10 mM taurine (10)	0.22 ± 0.01	0.78 ± 0.15[a,c]

Data are given as mean values ± SEM. k_1 constants were calculated for 22–30 min and k_2 for 40–50 min of superfusion. Taurine was present in incubation medium from the beginning of superfusion. Chemical ischemia and oxygen–glucose deprivation were evoked at the 31st minute of superfusion.
[a] $P < 0.05$ compared with k_1 of the same experimental group.
[b] $P < 0.05$ compared with k_2 during chemical ischemia.
[c] $P < 0.05$ compared with k_2 during oxygen–glucose deprivation. Number of independent experiments is given in parenthesis.

superfusion, and the efflux rate constants increased five-fold (Table 1, Fig. 1B).

Addition of taurine (5 mM or 10 mM) to medium at the beginning of superfusion did not alter the basal release rate or the fractional efflux rate constants of D-[^{3}H]aspartate efflux (Table 1). However, 10 mM taurine significantly reduced the release of D-[^{3}H]aspartate evoked by chemical ischemia or oxygen–glucose deprivation (Table 1, Figs. 1A, B, 2A, B). The lesser concentration of taurine (5 mM) did not influence the evoked release of D-[^{3}H]aspartate.

The roles of taurine transporter and GABA and glycine receptors were studied in the observed inhibitory effect of taurine on the release of D-[^{3}H]aspartate evoked by chemical ischemia. The GABA and glycine receptor blockers bicuculline (0.1 mM) and strychnine (0.1 mM) did not alter the basal efflux rate constants estimated for 22–30 min of superfusion. Likewise unaltered were the evoked efflux rate constants estimated for 40–50 min of superfusion in the presence of NaCN in glucose-free KRH (data not shown). Nor did these blockers prevent the inhibitory effect of 10 mM taurine on D-[^{3}H]aspartate release induced by chemical ischemia (Fig. 2A). The application of the taurine uptake inhibitor guanidinoethanesulfonate (GES) did not change the efflux rate of D-[^{3}H]aspartate during the basal experimental period and did not interfere with the effect of 10 mM taurine on the evoked D-[^{3}H]aspartate release (Fig. 2B). However, the application of GES itself diminished the release of D-[^{3}H]aspartate in chemical ischemia.

2.2. *Effects of D-aspartate and taurine on D-[^{3}H]aspartate release*

Incubation of corticostriatal slices with 0.5 mM unlabeled D-aspartate evoked D-[^{3}H]aspartate release, leading to a five-fold elevation in the efflux rate constant estimated for 34–50 min of superfusion (Table 2, Fig. 3). Addition of the competitive inhibitor of glutamate transporter DL-(+)-threo-3-hydroxyaspartate (THBA, 0.5 mM) during the basal period of superfusion enhanced the efflux rate, increasing the basal efflux rate constants three-fold. The slices pretreated with THBA and subjected thereafter to 0.5 mM D-aspartate in the absence of THBA released labeled D-aspartate at a slower rate than the slices incubated with D-aspartate, but not preloaded with THBA (Fig. 3). When THBA was present during the whole experimental period, the addition of D-aspartate to the incubation medium did not evoke any significant changes in the release of D-[^{3}H]aspartate when compared to the efflux rate constants of the basal release and of the release in the presence of D-aspartate only (Table 2). When the efflux constants are recalculated as percents of the basal release (k_1), the efflux rate constant in the presence of D-aspartate and THBA was significantly smaller than the efflux rate constant in the presence of D-aspartate alone. Addition of 10 mM taurine to the incubation medium did not affect the efflux of D-[^{3}H]aspartate evoked by D-aspartate (Fig. 3).

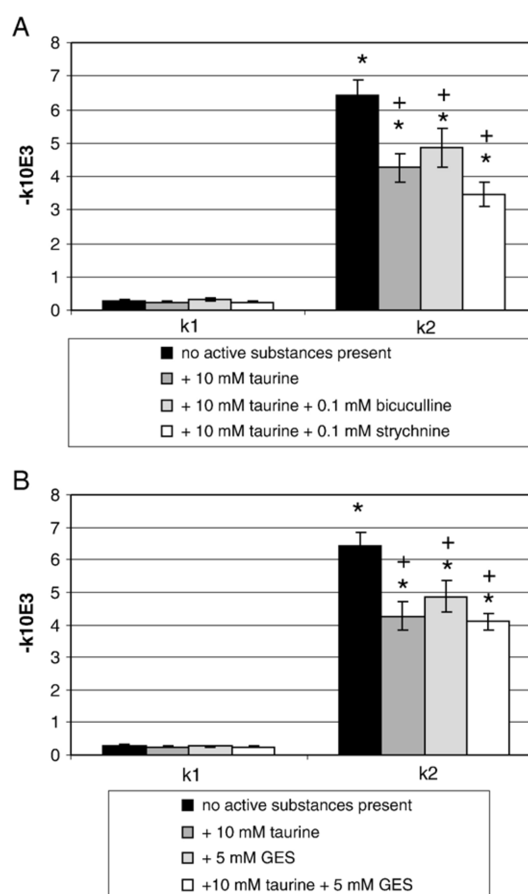

Fig. 2 – Rate constants of D-[^{3}H]aspartate release from corticostriatal slices under chemical ischemia, evoked by 0.5 mM NaCN in glucose-free KRH. k_1 was calculated for 22–30 min (basal conditions) and k_2 for 40–50 min of superfusion (chemical ischemia). (A) Effects of GABA$_A$ receptor antagonist bicuculline and glycine receptor antagonist strychnine and (B) effect of taurine uptake blocker GES. Data are given as mean values ± SEM of four to twelve experiments. Taurine, GES, strychnine or bicuculline was present in the incubation medium from the beginning of superfusion, and chemical ischemia was established at the 31st minute. *$P < 0.05$ compared with k_1 of the same experimental group; $^+P < 0.05$ compared with k_2 during chemical ischemia.

Table 2 – Rate constants of d-[3H]aspartate efflux from corticostriatal slices under homoexchange and heteroexchange conditions

Experimental conditions	Rate constants of basal release, $k_1 \times 10^3$ (22–30 min)	Rate constants of evoked release, $k_2 \times 10^3$ (34–50 min)	k_2, percents from k_1
D-aspartate during 31–50 min (12)	0.33 ± 0.04	1.57 ± 0.07 [a]	563.6 ± 68.6
THBA during 20–30 min (4)	1.07 ± 0.15 [b,c]	0.28 ± 0.01 [a,c]	–
THBA during 20–30 min; D-aspartate during 31–50 min (4)	1.06 ± 0.06 [b,c]	0.97 ± 0.08 [b,c]	–
THBA during 20–50 min (4)	1.38 ± 0.09 [b]	0.92 ± 0.14 [a,b,c]	–
THBA during 20–50 min; D-aspartate during 31–50 min (4)	1.16 ± 0.07 [b,c]	1.44 ± 0.25 [b]	119.4 ± 23.4 [d]

Data are given as mean values ± SEM. k_1 constants were calculated for 22–30 min and k_2 for 34–50 min of superfusion.
[a] $P < 0.05$ compared with k_1 of the same experimental group.
[b] $P < 0.05$ compared with k_1 of D-aspartate group (no active substances present during basal experimental period).
[c] $P < 0.05$ compared with k_2 of D-aspartate group (D-aspartate present).
[d] $P < 0.05$ compared with percents of k_2 of D-aspartate group. Number of independent experiments is given in parenthesis.

2.3. *Effects of hypo-osmotic medium and taurine on D-[^{3}H]aspartate release*

The D-[^{3}H]aspartate efflux rate was enhanced by the incubation of corticostriatal slices in hypo-osmotic medium in which NaCl was reduced by 50 mM. The calculated osmolarity of this solution was 234 mOsm. The rate constants of D-[^{3}H]aspartate release from the slices incubated in hypo-osmotic medium were two times higher than the constants under basal conditions (Fig. 4). This effect was attenuated by the volume-sensitive chloride channel blocker SITS. Two mM SITS did not change the efflux rate constants calculated for the basal period of superfusion, but markedly reduced them during the application of hypo-osmotic solution. The release of D-[^{3}H]aspartate evoked by the lowered osmolarity of the incubation medium was not attenuated by 10 mM taurine.

2.4. *Effects of veratridine and taurine on D-[^{3}H]aspartate release*

Activation of voltage-sensitive sodium channels by veratridine (0.1 mM) induced a considerable increase in the efflux rate of D-[^{3}H]aspartate (Fig. 5). Veratridine led to a 23-fold

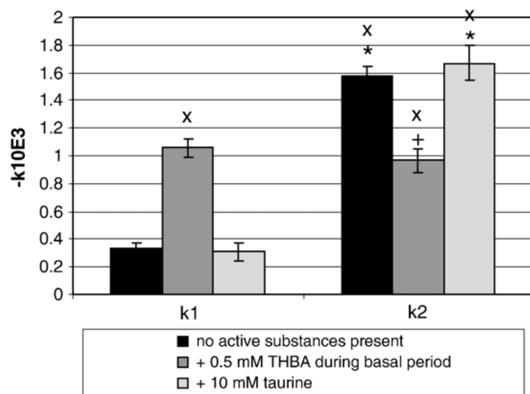

Fig. 3 – Rate constants of D-[^{3}H]aspartate release from corticostriatal slices under 0.5 mM d-aspartate application: the effects of the inhibitor of glutamate transporter THBA and taurine. k_1 was calculated for 22–30 min (basal conditions) and k_2 for 34–50 min of superfusion (0.5 mM D-aspartate). Data are given as mean values ± SEM of four to twelve experiments. Taurine was present in the incubation medium from the beginning of superfusion, and D-aspartate was applied at 31 min. THBA was present in the incubation medium during 0–30 min. ˣP < 0.05 compared with k_1 of D-aspartate group (no active compounds present in incubation medium during the period of calculation); *P < 0.05 compared with k_1 of the same experimental group; +P < 0.05 compared with k_2 during the application of unlabeled D-aspartate.

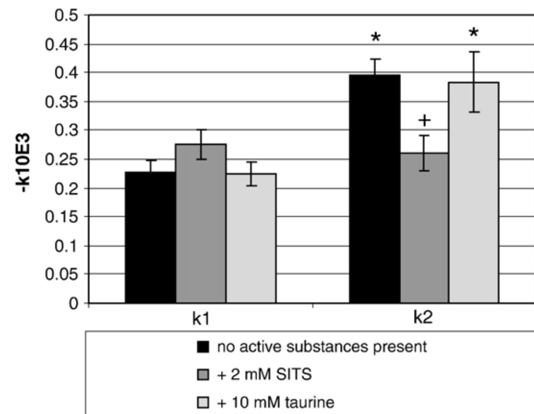

Fig. 4 – Rate constants of D-[^{3}H]aspartate release from corticostriatal slices under hypo-osmotic conditions: the effect of the volume-sensitive channel blocker SITS and taurine. Hypo-osmotic conditions were established by superfusing the slices in medium containing 76 mM NaCl instead of 126 mM. k_1 was calculated for 22–30 min (basal conditions) and k_2 for 34–50 min of superfusion (hypoosmotic conditions). Data are given as mean values ± SEM of four to twelve experiments. Taurine or SITS was present in the incubation medium from the beginning of superfusion, and hypo-osmotic conditions were established at the 31st minute. *P < 0.05 compared with k_1 of the same experimental group; +P < 0.05 compared with k_2 during the perfusion of hypo-osmotic medium without any active substances.

increase in the efflux rate constants when compared to the basal efflux. This increase was attenuated by the removal of Ca^{2+} from the incubation medium and preloading of THBA (0.5 mM) into the slices. In Ca^{2+}-free medium, the efflux rate constant of evoked D-[^{3}H]aspartate release was 45% of that of the evoked release in normal medium. THBA preloading induced a five-fold increase in the D-[^{3}H]aspartate efflux rate during the basal period, but lowered the evoked release. Taurine (10 mM) significantly diminished the release of D-[^{3}H] aspartate evoked by veratridine (Fig. 5).

3. Discussion

The release of glutamate under ischemic conditions was studied using D-[^{3}H]aspartate which is widely employed as a marker for L-glutamate release (Rutledge and Kimelberg, 1996; Roettger and Lipton, 1996; Saransaari and Oja, 1999; Dohovics et al., 2003; Marcoli et al., 2003). D-[^{3}H]Aspartate has been reported to be taken up by glial cells and nerve terminals but not by synaptic vesicles (Gunderson et al., 1995). However, biochemical studies suggest that this analog can be used as a marker for both cytoplasmic and vesicular pools of glutamate. In particular, D-[^{3}H]aspartate is shown to be released from synaptic terminals by high-potassium evoked depolarization, N-methyl-D-aspartate (NMDA) application and electric stimulation in a Ca^{2+}-dependent manner (Muzzolini et al., 1997; Savage et al., 2001; Bak et al., 2003). The release of glutamate and D-[^{3}H]aspartate from the cytosolic pool occurs in a similar manner (Roettger and Lipton, 1996; Saransaari and Oja, 1999). These findings validate the use of D-[^{3}H]aspartate as an indicator of glutamate release under most experimental conditions.

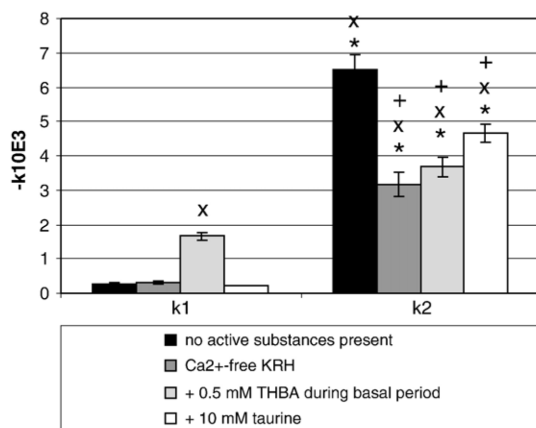

Fig. 5 – Rate constants of D-[^{3}H]aspartate release from corticostriatal slices under 0.1 mM veratridine application: the effects of Ca^{2+}-free medium, glutamate transporter inhibitor THBA and taurine. k_1 was calculated for 22–30 min (basal period) and k_2 for 34–50 min of superfusion (0.1 mM veratridine). Data are given as mean values ± SEM of four to twelve experiments. Taurine or Ca^{2+}-free medium was applied from the beginning of superfusion and veratridine at the 31st minute. THBA was present in incubation medium during 0–30 min. $^x P < 0.05$ compared with k_1 of the veratridine group (no active compounds present in incubation medium during the period of estimation); $^* P < 0.05$ compared with k_1 of the same experimental group; $^+ P < 0.05$ compared with k_2 during veratridine application.

To study the effect of taurine on ischemia-evoked glutamate release, two models of in vitro ischemia were used. Both experimental conditions inhibit glycolysis and oxidative metabolism and lead to excitotoxic cell death mediated by overactivation of glutamate receptors (Vornov, 1995). Chemical ischemia and oxygen–glucose deprivation induced a marked release of D-[^{3}H]aspartate, which was reduced by 10 mM taurine. The present paper is the first to report such an effect of taurine on the ischemic release of this glutamate analog. A rather high concentration of taurine was needed to elicit significant responses in D-[^{3}H]aspartate release. In this respect, taurine differs from other neuroactive amino acids. The extracellular concentration of taurine in the brain in vivo is about ten times higher than the concentrations of established amino acid neurotransmitters (Lerma et al., 1986; Miele et al., 1996; Molchanova et al., 2004a). The concentration of taurine in the synaptic cleft is probably still much higher than the overall extracellular concentration estimated by microdialysis. Ischemia evokes about a 30-fold increase in extracellular taurine concentration in vivo (Molchanova et al., 2004b). It means that the actual extracellular concentration under these conditions may reach at least the 1-mM range. In keeping with this, only relatively high concentrations of taurine have been found to be effective in other studies on different brain preparations (e.g., del Olmo et al., 2000b; Messina and Dawson, 2000; El Idrissi and Trenkner, 2003).

The inhibitor of taurine uptake GES did not interfere with the suppressive effect of taurine under ischemic conditions, which is in line with other studies showing no effect of taurine uptake inhibitors on the action of taurine (O'Byrne and Tipton, 2000; Chen et al., 2001; Louzada et al., 2004). However, GES attenuated D-[^{3}H]aspartate release similarly to taurine. GES has a number of other effects, including a mild agonistic effect at $GABA_A$ but not at $GABA_B$ receptors and an antagonistic effect at strychnine-sensitive glycine receptors (Sergeeva et al., 2002; Frosini et al., 2003a). These side effects hamper definitive establishment of the localization of taurine action on the basis of the experiments with GES.

Since taurine induces chloride currents across cell membranes (Oja et al., 1990; Belluzzi et al., 2004), the main molecular target of its action could be a chloride channel. Some taurine actions on neuronal preparations may be mediated by $GABA_A$ and glycine receptors (Malminen and Kontro, 1986; Kontro and Oja, 1987b; Ye et al., 1997; Belluzzi et al., 2004). However, neither bicuculline nor strychnine attenuated the effect of taurine on ischemia-evoked D-[^{3}H]aspartate release. The effects of taurine observed in this study are thus not likely to be mediated by the GABA- or glycine-governed chloride channels. Although taurine also binds to $GABA_B$ receptors (Kontro et al., 1990; Frosini et al., 2003a), their involvement is less likely. It has been shown that, in the presence of $GABA_A$ antagonists, GABA, but not taurine, evokes membrane hyperpolarization in hippocampal slices (del Olmo et al., 2000a). $GABA_B$ antagonists do not change the taurine-induced elevation in the membrane chloride conductance in

olfactory bulb slices (Belluzzi et al., 2004). These data indicate that taurine does not directly activate $GABA_B$ receptors.

Glutamate is released by several mechanisms during ischemia (Phillis and O'Regan, 2003). In the first minutes of ischemia, the energy sources are still not fully exhausted and glutamate release is activated by means of synaptic vesicular mechanisms triggered by membrane depolarization (Wahl et al., 1994). After total energy depletion, the subsequent efflux includes non-vesicular Ca^{2+}-independent release (Seki et al., 1999). It is mediated in part by Na^+-dependent amino acid transporters at plasma membranes operating in a reversed mode due to the ischemia-evoked ionic disbalance. The other non-vesicular glutamate release under ischemia is mediated by the swelling-induced opening of chloride channels. The above three means of glutamate release under ischemia have also been shown for D-[^{3}H]aspartate release (Roettger and Lipton, 1996; Saransaari and Oja, 1999; Marcoli et al., 2003). To determine which mechanism of ischemic D-[^{3}H]aspartate release is affected by taurine, the different modes of release were modeled pharmacologically.

Under hypo-osmotic conditions, cells swell and regulate their volume by extruding osmotically active substances. The amino acids released during this regulatory volume decrease include aspartate, taurine, glutamate, GABA and glycine (Estevez et al., 1999). Of these, taurine is considered the most efficacious osmolyte (Schaffer et al., 2000), and application of exogenous taurine has been shown to enhance cell volume regulation (Kreisman and Olson, 2003). We show that D-[^{3}H] aspartate is likewise released under hypo-osmotic conditions and the inhibitor of volume-activated chloride channels SITS was able to attenuate this release. However, we failed to show any effect of taurine on hypo-osmotically evoked D-[^{3}H] aspartate release, witnessing that the inhibitory effect of taurine on the ischemia-evoked D-[^{3}H]aspartate release is not likely to be mediated by volume regulatory mechanisms.

Glutamate is taken up by cells with glutamate transporters—a family of proteins which mediate the glutamate flux driven by sodium ion gradients (Nelson, 1998). In ischemia, due to energy failure, the ionic gradient is dissipated and the direction of action of amino acid transporters may be altered. It was shown that glutamate carrier blocker dihydrokainate suppresses glutamate release under global cerebral ischemia (Seki et al., 1999). To model this mode of glutamate release, we used the phenomenon of homoexchange, when exogenous application of the compound evokes the increase in extracellular concentration of the same compound preloaded into the tissue. Under these conditions, an extracellular compound both inhibits the reuptake and evokes a release of the preloaded substance (Bernath, 1992). In our experiments, D-[^{3}H]aspartate release was enhanced in the presence of unlabeled D-aspartate in the incubation medium. Uptake blockers can be used to prove the involvement of amino acid carriers in the release during homoexchange (Bernath, 1992). The inhibitors of glutamate transporters may theoretically deplete the intracellular pools of this amino acid due to the inhibition of reuptake. However, the vesicular (Bak et al., 2003) and osmotic (Rutledge and Kimelberg, 1996) modes of release were not affected by the glutamate uptake blockers. These evidences bespeak the absence of the depletion effect of uptake inhibitors on both vesicular and cytosolic glutamate pools labeled by D-[^{3}H]aspartate. The participation of the glutamate transporter in homoexchange-induced release was confirmed using the competitive glutamate transport inhibitor THBA. Because THBA is a competitive inhibitor, it needs to act from the same site as the intracellularly loaded D-[^{3}H] aspartate. The preloading of the competitive inhibitor before application of a stimulus will inhibit the release mediated by the reversed action of a transporter (Rutledge and Kimelberg, 1996). We show that THBA increases the extracellular concentration of D-[^{3}H]aspartate, probably by heteroexchange. The application of D-aspartate in the presence of this compound to the incubation medium did not evoke any significant elevation in the release of D-[^{3}H]aspartate. These data signify the involvement of glutamate transporters in the increase in D-[^{3}H]aspartate in the medium during D-aspartate application. However, to distinguish between the inhibition of reuptake and activation of release mediated by the transporter due to homoexchange, the inhibitor of the carrier should be present only intracellularly. THBA preloaded into the slices during the basal period of reperfusion and removed during the stimulation period also significantly inhibited the release of D-[^{3}H]aspartate evoked by homoexchange. This confirms that D-[^{3}H]aspartate release evoked by homoexchange is mediated by glutamate transporters. However, taurine did not interfere with this mode of D-[^{3}H]aspartate release.

Since taurine does not interact with the non-vesicular release of D-[^{3}H]aspartate evoked either by the reverse action of the transporters or by the opening of volume-activated chloride channels, one may conclude that the possible target of taurine action is the synaptic release. To check this alternative, a study was made of the effect of taurine on the D-[^{3}H]aspartate release evoked by depolarization using the activator of voltage-dependent sodium channels veratridine. This compound evoked a marked release of D-[^{3}H]aspartate, which was reduced under Ca^{2+}-free conditions and by THBA preloaded into the slices, indicating that under veratridine-evoked depolarization D-[^{3}H]aspartate efflux is mediated by both synaptic exocytosis and reversal of glutamate transporter. Taurine significantly lowered the release of D-[^{3}H]aspartate evoked by this mode. In this case, taurine may affect both vesicular- and transporter-mediated release of glutamate. However, we were able to show that taurine does not reduce the release evoked by homoexchange. Thus, our data indicate that the target of taurine effect may be either synaptic vesicular mechanisms directly or molecular events that lay upstream of the glutamate release reactions. It may be assumed that inhibitory effects of taurine on the ischemia-evoked D-[^{3}H]aspartate release are associated with the action of taurine on plasma membrane depolarization upstream of the glutamate release mechanisms. In line with this hypothesis is the evidence that the protective effect of taurine under excitotoxic conditions is mediated by hyperpolarization of plasma membranes, probably by activation of chloride channels (Wu et al., 2005).

In conclusion, the effect of exogenous taurine on the release of glutamate was studied using D-[^{3}H]aspartate as the non-metabolized analog of glutamate. It is shown that taurine suppresses the ischemia and depolarization-evoked release of D-[^{3}H]aspartate. This effect is not mediated by GABA and glycine receptors. At the same time, taurine does not have any

effect on the release evoked by the reversed action of glutamate transporters and hypo-osmotic conditions. The proposed site of taurine action is connected with either vesicular release reactions or mechanisms which regulate polarization of plasma membranes.

4. Experimental procedure

4.1. Materials

D-[³H]Aspartate (specific activity 1.48 PBq/mol) was purchased from Amersham International (Bristol, UK). Taurine was from Fluka (Buchs, Switzerland), and D-aspartate, DL-threo-β-hydroxyaspartate (THBA), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), veratridine, strychnine, bicuculline and GES were from Sigma (St. Louis, MO, USA). The liquid scintillation cocktail OptiPhase "SuperMix" was produced by Wallac Oy (Turku, Finland). Other chemicals were from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) and J.T. Baker (Deventer, Holland). All stock drug solutions were prepared in KRH, except for bicuculline, which was initially dissolved in dimethylsulfoxide (the final concentration of the solvent in the incubation medium was less than 0.01%).

4.2. *D-[³H]Aspartate efflux assay*

The experiments were carried out using adult NMRI mice (Orion, Espoo, Finland) of both sexes. Brain slices 0.4 mm thick, containing sensorimotor cortex and striatum, were prepared with a McIlwain tissue chopper on a cold plate. The slices were then transferred into preoxygenated KRH solution containing (mM) 126 NaCl, 1.3 $MgSO_4$, 1.3 NaH_2PO_4, 5.0 KCl, 0.8 $CaCl_2$, 15 HEPES and 10 D-glucose (pH 7.4 adjusted with NaOH, calculated osmolarity 334 mOsm) and loaded with D-[³H]aspartate (75 μM, 111 MBq/l, in the presence of 0.01 mM unlabeled D-aspartate). The preloading was performed during 30 min at 37 °C under O_2 atmosphere. Thereafter, the excessive label was washed out with warm oxygenated KRH and the slices placed in 1 ml chambers. The slices were superfused with KRH (eight chambers in parallel) delivered at the rate of 0.25 ml/min at 37 °C under O_2 atmosphere. The first fraction of superfusate was collected during 20 min, and this experimental period was regarded as the recovery period. Thereafter, fractions were collected every 2 min. First five samples were collected for estimation of the basal efflux rate of D-[³H]aspartate. The next ten 2-min fractions were collected for the estimation of the evoked efflux of D-[³H]aspartate. Taurine and other inhibitors were already present from the beginning of superfusion. In some experimental groups, THBA was loaded into the slices during the recovery and basal efflux periods and was excluded from incubation medium during the measurement of evoked release. At the end of superfusion, the slices were homogenized in 5% trichloracetic acid and centrifuged. Aliquots of the supernatant and superfusate fractions were mixed with the liquid scintillation cocktail and subjected to radioactivity measurement in a liquid scintillation counter, 1450 Microbe PLUS (Wallac Oy, Turku, Finland). The counts were corrected for quenching with the aid of external standardization, using Wallac 1450 MicroBeta 4.0 software.

4.3. *Data analysis*

Based on the raw experimental data, two parameters of D-[³H]aspartate efflux were calculated: the percentage of D-[³H]aspartate released per minute and the fractional efflux rate

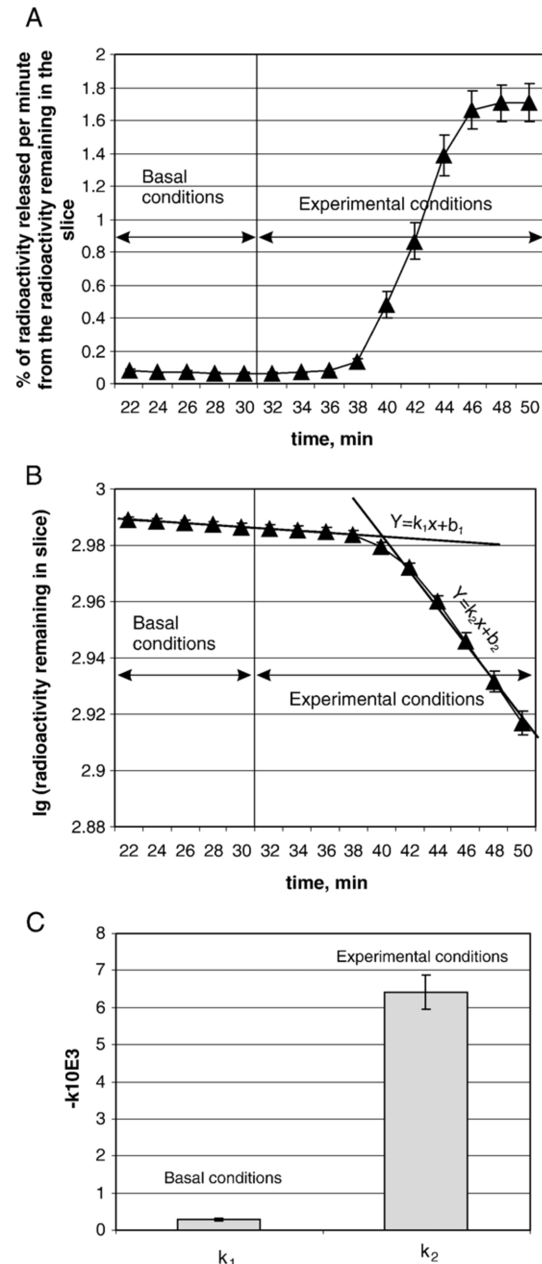

Fig. 6 – D-[³H]aspartate release under basal conditions and during chemical ischemia: an example of the data calculations. (A) Percentage of the radioactivity released per minute from the radioactivity remaining in slices, (B) logarithm of the radioactivity remaining in slices (parts per thousand from the total radioactivity) and (C) efflux rate constants for the basal and experimental periods. Data are given as mean values ± SEM of 12 experiments. Chemical ischemia and oxygen–glucose deprivation were evoked at the 31st minute of superfusion; k_1 was calculated for 22–30 min and k_2 for 40–50 min of superfusion.

constants. The total amount of D-[^{3}H]aspartate in a slice at the beginning of an experiment and the amount of label in the slice during each interval of fraction collection were calculated from the radioactivity in superfusate fractions and from the amount of label remaining in the slice at the end of the experiment. The rate of D-[^{3}H]aspartate release during the period of fraction collection was computed as percentage of radioactivity released in 1 min from the total amount of label in the slice (Fig. 6A). This parameter shows the speed of D-[^{3}H] aspartate release during any short period of experiment. The fractional efflux rate constants were calculated for different longer experimental periods as the negative slopes for the regression lines of the logarithm of radioactivity remaining in a slice against the superfusion time (Figs. 6B, C). The coefficient of correlation for the regression line, r, was in all cases greater than 0.95. This parameter characterizes long-term changes in the efflux rate. The rate constants were determined for the prestimulation period (basal release, k_1) and for the stimulation period (evoked release, k_2). The period chosen for calculation was selected on the basis of good linearity of the function. Both efflux rates and efflux rate constants are presented as mean values ± SEM for four to twelve experiments. Statistical comparison was made using the two-tailed Student's t test. The level of statistical significance (P) was set at 0.05.

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Inhibitory effect of taurine on veratridine-evoked D-[^{3}H]aspartate release from murine corticostriatal slices: involvement of chloride channels and mitochondria

Svetlana M. Molchanova[1,3], Simo S. Oja[2], and Pirjo Saransaari[1]

[1] University of Tampere, Medical School, Brain Research Center, Tampere, Finland

[2] Tampere University Hospital, The Centre for Laboratory Medicine, Tampere, Finland

[3] I.M. Sechenov Institute of Experimental Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia

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Corresponding author: Svetlana M. Molchanova

Postal address: Medical School, 33014 University of Tampere, Finland

Tel.: +358 3 3551 7320

Fax: +358 3 3551 6170

e-mail: Svetlana.Molchanova@uta.fi

We have previously shown that the inhibitory neuromodulator taurine attenuates the release of preloaded D-[^{3}H]aspartate from murine corticostriatal slices evoked by ischemic conditions or by application of the sodium channel agonist veratridine. The release of D-[^{3}H]aspartate (a non-metabolized analog of glutamate) was used as an index of glutamate release. The aim of the present study was to reveal the molecular mechanisms responsible for this inhibitory effect of taurine. It was shown that 10 mM taurine suppresses D-[^{3}H]aspartate release evoked by 0.1 mM veratridine, but does not affect the high-K^+- (50 mM) or ouabain- (0.1 mM) evoked release. Taurine had no effect in Ca^{2+}-free medium when the synaptic exocytosis of D-[^{3}H]aspartate was inhibited. Nor did it suppress the release from slices preloaded with the competitive glutamate uptake blocker DL-threo-β-hydroxyaspartate (THBA), which inhibits D-[^{3}H]aspartate release mediated by the reverse action of glutamate transporters. Omission of Cl^- from the incubation medium reduced the effect of taurine, signifying the involvement of a Cl^- channel. The glycine receptor antagonist strychnine and the $GABA_A$ receptor antagonist bicuculline did not block the taurine effect, although picrotoxin, a less specific blocker of agonist-gated chloride channels, completely prevented the effect of taurine on veratridine-induced D-[^{3}H]aspartate release. The respiratory chain blocker rotenone or mitochondrial protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in combination with the mitochondrial ATPase inhibitor oligomycin, which inhibit the mitochondrial Ca^{2+} uniporter, also reduced the effect of taurine. The results obtained in the present study show that taurine acts specifically on the release of preloaded D-[^{3}H]aspartate evoked by veratridine, but not on that evoked by other depolarizing agents, and affects the release mediated both by synaptic exocytosis and the reverse action of glutamate transporter. Taurine may attenuate D-[^{3}H]aspartate release by regulation of mitochondrial Ca^{2+} sequestration and by activation of a chloride channel, but not that governed by $GABA_A$ or strychnine-sensitive glycine receptors.

Section: Neurophysiology, Neuropharmacology and other forms of Intercellular Communication

Keywords: Taurine Glutamate Corticostriatal slices Agonist-gated chloride channels Mitochondria

Abbreviations: CCCP, carbonyl cyanide 3-chlorophenylhydrazone; GABA, γ-aminobutyric acid; KRH, Krebs-Ringer-HEPES buffer; TAG, 6-aminomethyl-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1dioxide; THBA, DL-threo-β-hydroxyaspartate

1. Introduction

Taurine (2-aminoethanesulphonic acid) is a sulfur-containing amino acid abundant in the central nervous system of mammals (Huxtable, 1989). It modulates neuronal activity, participates in osmoregulation, regulates cellular Ca^{2+} fluxes, and affects protein phosphorylation and membrane ion channels (Lombardini, 1994; Shaffer et al., 2000; Foos and Wu, 2002; El Idrissi and Trenkner, 2004; Albrecht and Schousboe, 2005). Taurine is also thought to act as an inhibitory neuromodulator (Oja and Saransaari, 1996; El Idrissi and Trenkner, 2004; Albrecht and Schousboe, 2005). However, the cellular mechanisms underlying neuroactive taurine actions remain unclear.

Regulation of fluxes and intracellular levels of Ca^{2+} mediates many neuroprotective effects of taurine. It improves cell survival upon application of toxic concentrations of glutamate by lowering intracellular free Ca^{2+} via reduction of the glutamate-evoked Ca^{2+} influx into cells and Ca^{2+} release from intracellular storages (El Idrissi and Trenkner, 1999; Zhao et al., 1999; Chen et al., 2001; Foos and Wu, 2002; El Idrissi and Trenkner, 2004; Wu et al., 2005). This latter effect may be connected to the regulation of Ca^{2+} sequestration by mitochondria, which are the main intracellular Ca^{2+} storage pools. Indeed, taurine affects Ca^{2+} concentrations in isolated brain and liver mitochondria and cultured cerebellar granule cells (Li and Lombardini, 1991; Palmi et al., 1999; El Idrissi and Trenkner, 2003).

Taurine has been shown to be a partial agonist at inhibitory amino acid receptors (Oja and Saransaari, 1996; El Idrissi and Trenkner, 2004; Albrecht and Schousboe, 2005). Indeed, application of taurine enhances Cl^- influx in different preparations, which effect is inhibited by $GABA_A$ receptor antagonists (Oja et al., 1990; Belluzzi et al., 2004; El Idrissi and Trenkner, 2004). The ability of taurine to depress evoked field responses has also been attributed to the activation of $GABA_A$ and strychnine-sensitive glycine receptors (Chepkova et al., 2002) and their involvement has also been shown in other studies (O'Burne and Tipton, 2000; Louzada et al., 2004; Hilgier et al., 2005). It is not yet clear whether taurine only exerts its inhibitory effects through GABA and glycine receptors due to the structural similarity of these amino acids or whether separate taurine receptors are involved. If the former assumption holds then there should exist a strict compartmentalization of the extracellular pools of GABA, glycine and taurine, especially in view of the very high extracellular concentrations of taurine in vivo. The possible presence of a specific taurine receptor in mammalian nervous tissue has been considered. A putative specific taurine recognition site in synaptic membranes has been described (Kontro and Oja, 1987; Wu et al., 1992; Wu et al., 2001; Frosini et al., 2003a,b), which may also participate in the molecular reaction chain leading to intracellular Ca^{2+} regulation by taurine (Foos and Wu, 2002; Wu et al., 2005). Using rabbit brain membranes, four taurine analogs, including 6-

aminomethyl-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (TAG), have been shown to specifically bind to the taurine binding site but not to the $GABA_A$ and $GABA_B$ receptors (Frosini et al., 2003a). Three of these compounds also antagonized taurine effects on body temperature (Frosini et al., 2003b). Furthermore, the specific antagonist of taurine (TAG) selectively inhibits a number of physiological and biochemical effects of taurine, without significantly affecting the GABAergic or glycinergic systems (Martin et al., 1981; Girard et al., 1982; Okamoto et al., 1983; Wessberg et al., 1983; Kubo et al., 1993; Engelmann et al., 2001). All these findings bespeak the existence of a specific taurine receptor.

The aim of the present study was to shed light on the mechanisms of the inhibitory effects of taurine in mammalian nervous tissue. Taurine inhibits the release of D-[^{3}H]aspartate (a non-metabolized analog of glutamate) from ischemic and depolarized corticostriatal slices (Molchanova et al., 2006), a model which was also used here. In particular, we studied the involvement of agonist-gated Cl^- channels and regulation of intracellular Ca^{2+} concentration by mitochondria in the taurine-induced suppression of veratridine-evoked D-[^{3}H]aspartate release. Based on the results obtained, the possible existence of a specific taurine receptor is discussed.

2. Results

The voltage-gated Na^+ channel opener veratridine (0.1 mM) evoked a significant release of preloaded D-[^{3}H]aspartate from incubated murine corticostriatal slices (Fig. 1). The maximal release was 1.99±0.14 % of the radioactivity in slices per minute 10 min after veratridine administration. This release was inhibited by preincubation of the slices with 10 mM taurine. The inhibition was maximal within 10 min after veratridine administration (68 % of D-[^{3}H]aspartate release in the presence of veratridine only). Other depolarizing agents, 50 mM KCl and the Na^+, K^+-ATPase inhibitor ouabain (0.1 mM) also evoked D-[^{3}H]aspartate release, though to a lesser extent than veratridine (Fig. 1). The maximal D-[^{3}H]aspartate release evoked by high K^+ concentrations was encountered within 10 min after 50 mM KCl perfusion (0.59±0.06 % of the radioactivity in slices per minute). Ouabain evoked a gradual increase in D-[^{3}H]aspartate release to 1.03±0.19 % within 16 min. Taurine did not attenuate the D-[^{3}H]aspartate release evoked by the high K^+ concentrations and ouabain (Fig. 1).

The veratridine-induced release of D-[^{3}H]aspartate was significantly reduced in Ca^{2+}-free Krebs-Ringer-HEPES buffer (KRH). D-[^{3}H]Aspartate release under these conditions was 39-58 % of the veratridine-induced release in normal KRH (Fig. 2A). The release of D-[^{3}H]aspartate evoked by veratridine was also inhibited in slices preloaded with the glutamate transporter blocker DL-threo-β-hydroxyaspartate (THBA), though 0.5 mM THBA enhanced D-[^{3}H]aspartate release during the basal experimental period (Fig. 2B). After the removal of THBA from the

incubation medium, the release of D-[^{3}H]aspartate diminished to the basal level (data not shown). However, when these slices were exposed to veratridine, D-[^{3}H]aspartate release was significantly smaller (the maximal decrease was 49 % of the release from slices not preloaded with THBA, Fig. 2B). The fraction of D-[^{3}H]aspartate release not inhibited by Ca^{2+} omission or THBA preloading was not sensitive to taurine (Fig. 2A,B).

Incubation of corticostriatal slices in Cl^--free medium markedly enhanced both basal and veratridine-evoked D-[^{3}H]aspartate release (Fig. 3), the maximal release under these conditions being 3.1±0.12 % of the radioactivity in slices. Taurine reduced the veratridine-evoked release in the absence of Cl^- ions, but less (86-88 % of the evoked release in Cl^--free KRH) than in normal KRH (68-78 %, Fig. 3).

The glycine receptor blocker strychnine (10 μM) did not affect the veratridine-induced D-[^{3}H]aspartate release either in the absence or in the presence of taurine (Fig. 4A). Nor did the inhibitor of $GABA_A$ receptors bicuculline (10 μM, Fig. 4B). A higher concentration of these substances (100 μM) likewise exerted no effect (data not shown). However, a less specific blocker of both glycine and $GABA_A$ receptors, picrotoxin (50 μM), completely prevented the inhibitory effect of taurine on the veratridine-evoked D-[^{3}H]aspartate release (Fig. 4C), while not altering the [^{3}H]aspartate release evoked by veratridine.

The inhibitor of the mitochondrial electron transport chain rotenone (20 μM) in combination with the inhibitor of mitochondrial ATPase oligomycin (15 μM) increased the release of D-[^{3}H]aspartate during both basal and subsequent evoked experimental periods (Fig. 5A), leading to a maximal release of 3.07±0.2 % of the radioactivity in slices. Mitochondrial protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 2 μM), applied with oligomycin, had the same enhancing effect on D-[^{3}H]aspartate release (Fig. 5B). In the presence of taurine and under inhibition of mitochondrial function by rotenone, D-[^{3}H]aspartate release was slower 8 min after veratridine administration (76 % of the release in the absence of taurine). However, the release was not affected at any other experimental time point (Fig. 5A). In the presence of CCCP taurine did not have the inhibitory effect on D-[^{3}H]aspartate release at any experimental time point (Fig. 5B).

3. Discussion

Our previous study showed that taurine attenuates the depolarization-dependent component of ischemia-evoked release of D-[^{3}H]aspartate from incubated corticostriatal slices (Molchanova et al., 2006), in which context the use of D-[^{3}H]aspartate as the non-metabolized analog of glutamate was also discussed. The present findings demonstrate the suppressive effect of taurine on the veratridine-evoked release of preloaded D-[^{3}H]aspartate. Taurine is also known

to inhibit the release of neurotransmitters, in particular, that of glutamate (Kamisaki et al., 1993; Zheng 2001). However, taurine did not affect the D-[^{3}H]aspartate release evoked by high K^+ concentrations and the Na^+, K^+-ATPase inhibitor ouabain. A link between the putative taurine molecular target and voltage-gated sodium channels may thus exist. Taurine alters the properties of tetrodotoxin-sensitive Na^+ currents in rat dorsal root ganglion neurons (Yu et al., 2005). This effect may be related to the action of taurine as the charged substance on the lipid surroundings of the Na^+ channels or to interactions with a site allosterically coupled to the Na^+ channel subunits. Taurine may also influence the state of membrane polarization, increasing membrane Cl^- conductance (Oja et al., 1990; Belluzzi et al., 2004; El Idrissi and Trenkner, 2004) and thus antagonize the depolarization evoked by activation of Na^+ channels.

Depolarizing agents evoke glutamate release through different pathways, including release by synaptic exocytosis and release mediated by the reverse action of transporters. Omission of Ca^{2+} from the incubation medium inhibits the synaptic release pathway. Preloading of the glutamate transport inhibitor THBA suppresses the glutamate release mediated by the reversal of carriers evoked by the disturbed membrane gradient of Na^+. We have shown that both Ca^{2+}-free medium and THBA preloaded into the slices suppress veratridine-evoked D-[^{3}H]aspartate release, signifying that veratridine-evoked depolarization leads to glutamate release through both pathways (Molchanova et al., 2006). In the presence of THBA inside the slices or under Ca^{2+} depletion taurine did not now affect veratridine-evoked D-[^{3}H]aspartate release. Hence, when the release by synaptic exocytosis or by the reverse action of glutamate transporters is blocked, taurine does not inhibit the remaining D-[^{3}H]aspartate release. Both release pathways are activated by the depolarization of neuronal membrane. We may assume that taurine inhibits membrane depolarization and thus reduces glutamate release mediated by both release pathways. This hypothesis is in accordance with the findings of Wu and colleagues (2005), who have shown that taurine reduces glutamate-evoked Ca^{2+} influx and that this effect is non-specific and independent of the type of Ca^{2+} channel. Taurine may hyperpolarize cell membranes and affect voltage-dependent Ca^{2+} influx into the cell. Such a conception is corroborated by the finding that taurine indeed impedes glutamate-induced membrane depolarization (Wu et al., 2005).

Taurine may evoke hyperpolarization of plasma membranes by affecting the membrane Cl^- conductance. While Cl^- omission enhanced both basal and veratridine-evoked D-[^{3}H]aspartate release, the effect of taurine under these conditions was reduced. These results confirm the involvement of some Cl^- channels in the mechanism underlying the inhibitory effect of taurine. The specific inhibitors of $GABA_A$ and glycine receptors, bicuculline and strychnine, respectively, did not alter the effect of taurine on veratridine-evoked D-[^{3}H]aspartate release. These results are at variance with previous findings suggesting that these types of receptors

mediate most of the inhibitory effects of taurine (O'Byrne and Tipton, 2000; Louzada et al., 2004). However, the less specific inhibitor of these receptors picrotoxin at a concentration of 50 μM completely prevented the taurine effect. Similar effects of Cl^- channel blockers have also been reported in other studies. In an in vivo model of ammonia neurotoxicity, picrotoxin significantly reduced the protective effect of taurine, while bicuculline and strychnine were less effective (Hilgier et al., 2005). Vasopressin release induced by taurine from the rat neurohypophysis is blocked by picrotoxin, but not by bicuculline or strychnine (Song and Hatton, 2003). The convulsant alkaloid picrotoxin is an equimolar mixture of picrotoxinin and picrotin, and is regarded as a channel blocker for $GABA_A$ receptors, $GABA_C$ receptors and α-homomeric glycine receptors (Jentsch et al., 2002; Lynch, 2004). Since the selective antagonists of $GABA_A$ and glycine receptors were shown here to be ineffective in preventing the inhibitory effect of taurine, the effect of picrotoxin is not related to the inhibition of these types of chloride channels. As $GABA_C$ receptors are also sensitive to picrotoxin but practically not gated by taurine (Jentsch et al., 2002; Martínez-Torres and Miledi, 2004), their involvement in the inhibitory effect of taurine is also questionable.

The ability of picrotoxin to prevent the suppressive effect of taurine on the evoked D-[^{3}H]aspartate release may be related to activation of the putative taurine receptor. If this was the case, then the putative taurine receptor should be deprived of the binding sites for bicuculline and strychnine but possesses the picrotoxin-binding site. Picrotoxin sensitivity is apparently determined by the presence of threonine in the 6' position of the MT2 domain, which forms pore walls in $GABA_A$ and glycine α subunits (Lynch, 2004). It is tempting to speculate that the structure of the taurine receptor may be very similar to the GABA and glycine-gated chloride channels, with maximal homology in the protein domains which form the channel pore. It is interesting to note that while taurine usually demonstrates low affinity for the native GABA or glycine receptors, certain point mutations substantially increase its potency to activate the channels associated with these receptors (Martínez-Torres and Miledi, 2004; Miller et al., 2004).

Taurine is also thought to exert its action through regulation of intracellular Ca^{2+} concentrations by affecting Ca^{2+} storage pools. Taurine activates the accumulation of $^{45}Ca^{2+}$ in mitochondria isolated from rat liver or cortical nerve endings (Li and Lombardini, 1991; Palmi et al., 1999). This effect would appear to be related to the action of taurine on the mitochondrial uniporter, since taurine does not alter the Ca^{2+} release evoked by ruthenium red (Palmi et al., 1999) and does not prevent the opening of permeability transition (Palmi et al., 2000). The mitochondrial Ca^{2+} uptake is mediated by the mitochondrial uniporter and driven by the membrane potential (Duchen, 1999, Nicholls and Budd, 2000). One of the experimental approaches to inhibit the action of the mitochondrial uniporter includes the use of mitochondrial

inhibitors, which induce a collapse of the mitochondrial membrane potential (Khodorov et al., 1999; Billups and Forsythe, 2002). To check the involvement of mitochondrial Ca^{2+} uptake in the inhibitory effects of taurine on evoked D-[^{3}H]aspartate release, we blocked this uptake by the respiratory chain blocker rotenone, protonophore CCCP and the mitochondrial ATPase inhibitor oligomycin. The latter prevents the fast ATP depletion caused by the reversed operation of mitochondrial ATP synthase. Rotenone and oligomycin have been shown to prevent Ca^{2+} uptake by mitochondria under excitotoxic conditions and after short-term depolarization (Khodorov et al., 1999; Billups and Forsythe, 2002). CCCP in combination with oligomycin also prevents Ca^{2+} clearance by mitochondria from intracellular space (Babcock and Hille, 1998; Dykens et al., 2002). In our study, they enhanced D-[^{3}H]aspartate release under both basal conditions and during veratridine administration. However, in the presence of these inhibitors the suppressive effect of taurine on D-[^{3}H]aspartate release was significantly diminished or absent. These results bespeak the involvement of regulation of cellular Ca^{2+} homeostasis in the mechanisms of the taurine-induced inhibition of glutamate release. However, the question remains open whether the activation of picrotoxin-dependent chloride fluxes and mitochondrial Ca^{2+} regulation are in a single chain of taurine-activated molecular reactions or are independent sites of action.

To conclude, we clearly demonstrated inhibition of the veratridine-evoked D-[^{3}H]aspartate release by taurine. In contrast, taurine did not inhibit ouabain- or high KCl-evoked release. The effect of taurine on the veratridine-evoked D-[^{3}H]aspartate release was not discernible upon inhibition of the synaptic and transporter-mediated release pathways. Both Cl^- omission from the incubation medium and inhibition of the mitochondrial Ca^{2+} uptake reduced the effect of taurine. The specific blockers of $GABA_A$ and glycine receptors bicuculline and strychnine were ineffective, while the less specific ligand-gated Cl^- channel blocker picrotoxin completely prevented the taurine-evoked inhibition of D-[^{3}H]aspartate release. We suggest that taurine may reduce depolarization-evoked glutamate release through regulation of both mitochondrial Ca^{2+} uptake and plasma membrane polarization, probably by activating some picrotoxin-sensitive ligand-gated chloride channel.

4. Experimental procedure

D-[^{3}H]Aspartate (specific activity 1.48 PBq/mol) was purchased from Amersham International (Bristol, UK). Taurine was from Fluka (Buchs, Switzerland) and D-aspartate, THBA, veratridine, strychnine, bicuculline, picrotoxin, rotenone, CCCP and oligomycin from Sigma (St. Louis, MO, USA). The liquid scintillation cocktail OptiPhase "SuperMix" was produced by Wallac Oy (Turku, Finland). Other chemicals were from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) and J.T.Baker (Deventer, Holland). All stock drug solutions were

prepared in KRH, except for bicuculline, rotenone, CCCP and oligomycin, which were initially dissolved in dimethylsulfoxide (the final concentration of the solvent in the incubation medium did not exceed 0.01 %).

The experiments were carried out using adult NMRI mice (Orion, Espoo, Finland) of both sexes, according to the procedure previously described (Molchanova et al., 2006). There was no gender difference in the results. Briefly, brain slices 0.4 mm thick, containing sensorimotor cortex and striatum, were preincubated for 30 min with D-[^{3}H]aspartate (75 µM**,** 111 MBq/l, in the presence of 0.01 mM unlabeled D-aspartate) in preoxygenated KRH solution containing (mM) 126 NaCl, 1.3 $MgSO_4$, 1.3 NaH_2PO_4, 5.0 KCl, 0.8 $CaCl_2$, 15 HEPES and 10 D-glucose (pH 7.4 adjusted with NaOH). Two to four slices were prepared from one animal. Thereafter, the slices were placed in 1 ml chambers and superfused with KRH delivered at a rate of 0.25 ml/min at 37°C under O_2 atmosphere. The first fraction of superfusate was collected within 20 min, and this experimental period was regarded as the recovery period. Thereafter, fractions were collected every 2 min. At the end of superfusion, the slices were homogenized in 5% trichloracetic acid and centrifuged. Aliquots of the supernatant and superfusate fractions were mixed with the liquid scintillation cocktail and subjected to radioactivity measurement in a liquid scintillation counter, 1450 Microbeta PLUS (Wallac Oy, Turku, Finland). The counts were corrected for quenching with the aid of external standardization, using Wallac 1450 MicroBeta 4.0 software.

The total amount of D-[^{3}H]aspartate in a slice at the beginning of an experiment and the amount of label in the slice during each interval of fraction collection were calculated from the radioactivity in superfusate fractions and from the amount of label remaining in the slice at the end of the experiment. The rate of D-[^{3}H]aspartate release was computed as a percentage of radioactivity released in one minute from the total amount of label in the slice. Data are presented as mean values ± SEM for four to sixteen experiments. Statistical comparison was made using the two-tailed Student *t*-test. The level of statistical significance (*p*) was set at 0.05.

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FIGURE LEGENDS

Fig. 1. Release of D-[^{3}H]aspartate from corticostriatal slices under depolarizing conditions and in the presence of taurine (10 mM). Depolarization was evoked by application of 0.1 mM veratridine, 0.1 mM ouabain or 50 mM KCl. Fifty mM KCl was added to medium while the NaCl concentration was reduced to keep the osmolarity constant. Data are given as mean values ± SEM of four to twelve experiments. * $P < 0.05$ (experimental group 1 versus experimental group 2); $^{\times}$ $P < 0.05$ (experimental group 1 versus experimental group 3; experimental group 1 versus experimental group 5); $^{+}$ $P < 0.05$ (experimental group 1 versus experimental group 3). The statistical difference between experimental points of the following groups obtains, but is not shown in figure: group 1 versus group 4, group 1 versus group 6, group 2 versus group 3, group 2 versus group 4, group 2 versus group 5, group 2 versus group 6.

Fig. 2. Release of D-[^{3}H]aspartate from corticostriatal slices under veratridine (0.1 mM) and taurine (10 mM) application: effects of Ca^{2+}-free medium (A) and 0.5 mM THBA (B). In Ca^{2+}-free medium $CaCl_2$ was substituted by an equimolar amount of choline chloride. Data are given as mean values ± SEM of four to twelve experiments. * $P < 0.05$ (experimental group 1 versus experimental group 2); $^{\times}$ $P < 0.05$ (experimental group 1 versus experimental group 3). The statistical difference between experimental points of the following groups obtains, but is not shown in figure: (A) group 1 versus group 4, group 2 versus group 3, group 2 versus group 4; (B) group 1 versus group 4, group 2 versus group 3, group 2 versus group 4.

Fig. 3. Release of D-[^{3}H]aspartate from corticostriatal slices under veratridine (0.1 mM) and taurine (10 mM) application: effect of Cl^--free medium. In Cl^--free medium NaCl, KCl and $CaCl_2$ were substituted by equimolar amounts of corresponding acetate salts. Data are given as mean values ± SEM of twelve experiments. * $P < 0.05$ (experimental group 1 versus experimental group 2); $^{\times}$ $P < 0.05$ (experimental group 1 versus experimental group 3); $^{+}$ $P < 0.05$ (experimental group 3 versus experimental group 4). The statistical difference between experimental points of the following groups obtains, but is not shown in figure: group 1 versus group 4, group 2 versus group 3, group 2 versus group 4.

Fig. 4. Release of D-[^{3}H]aspartate from corticostriatal slices under veratridine (0.1 mM) and taurine (10 mM) application: effects of 10 µM strychnine (A), 10 µM bicuculline (B) and 50 µM picrotoxin (C). Data are given as mean values ± SEM of six to twelve experiments. * $P < 0.05$ (experimental group 1 versus experimental group 2); $^{\times}$ $P < 0.05$ (experimental group 3

versus experimental group 4); $^{+}$ $P < 0.05$ (experimental group 2 versus experimental group 4). The statistical difference between experimental points of the following groups obtains, but is not shown in figure: (A) group 1 versus group 4, group 2 versus group 3; (B) group 1 versus group 4, group 2 versus group 3; (C) group 2 versus group 3.

Fig. 5. Release of D-[^{3}H]aspartate from corticostriatal slices under veratridine (0.1 mM) and taurine (10 mM) application: effect of 20 μM rotenone and oligomycin (15 μg/ml, A) and 2 μM CCCP and oligomycin (15 μg/ml, B). Data are given as mean values ± SEM of seven to twelve experiments. * $P < 0.05$ (experimental group 1 versus experimental group 2); $^{\times}$ $P < 0.05$ (experimental group 1 versus experimental group 3); $^{+}$ $P < 0.05$ (experimental group 3 versus experimental group 4). The statistical difference between experimental points of the following groups obtains, but is not shown in figure: group 1 versus group 4, group 2 versus group 3, group 2 versus group 4.

Figure 1

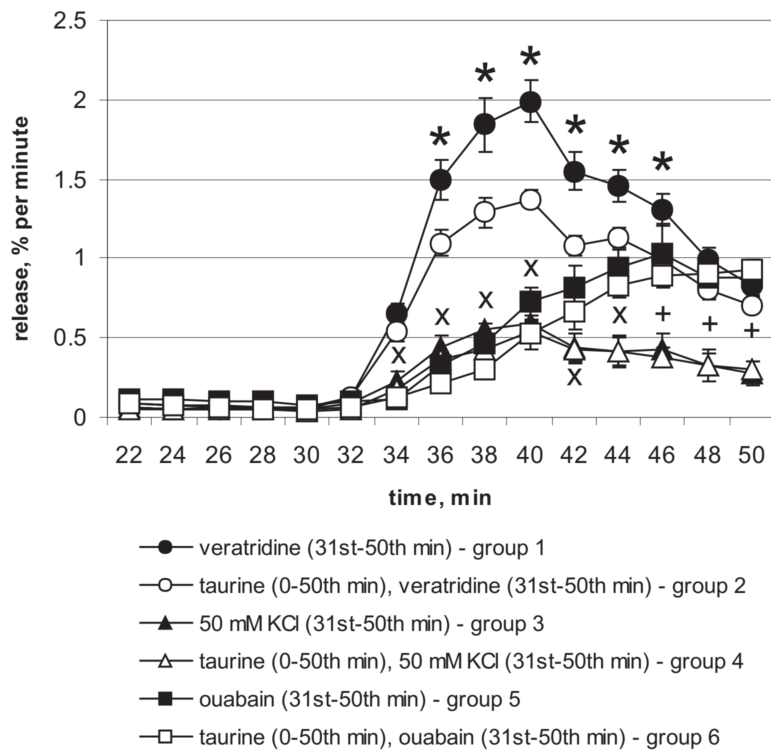

Figure 2A

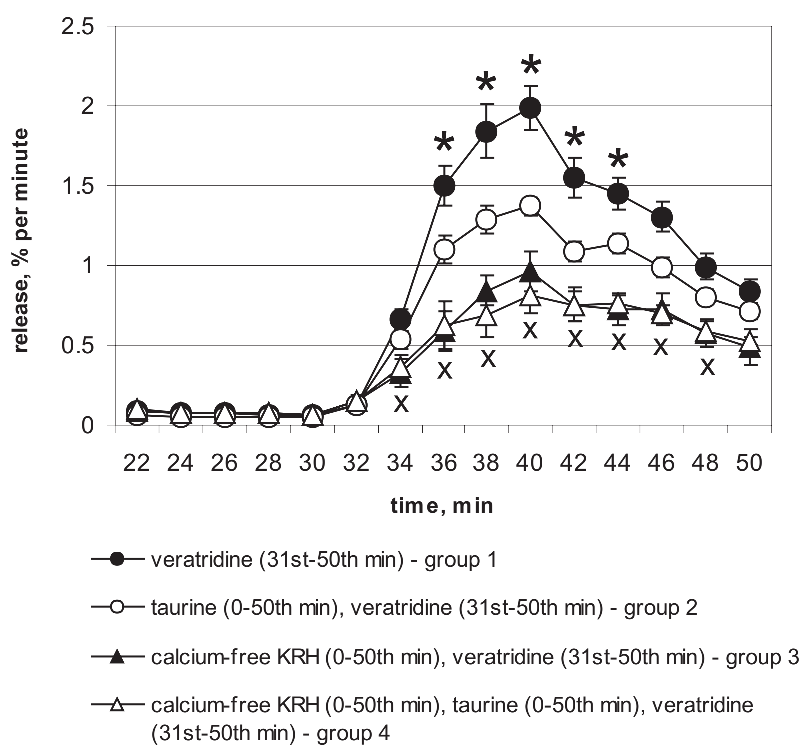

Figure 2B

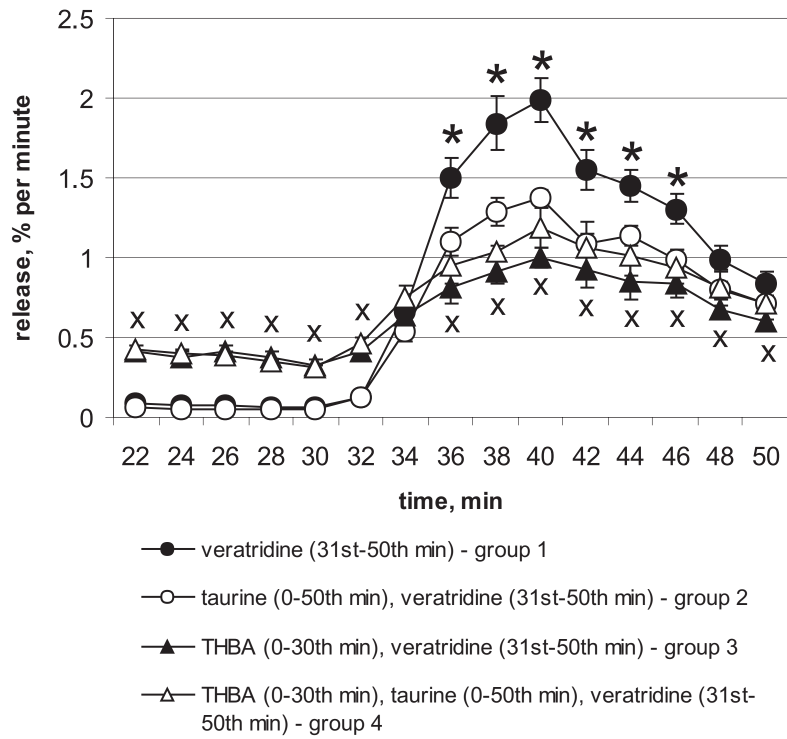

Figure 3

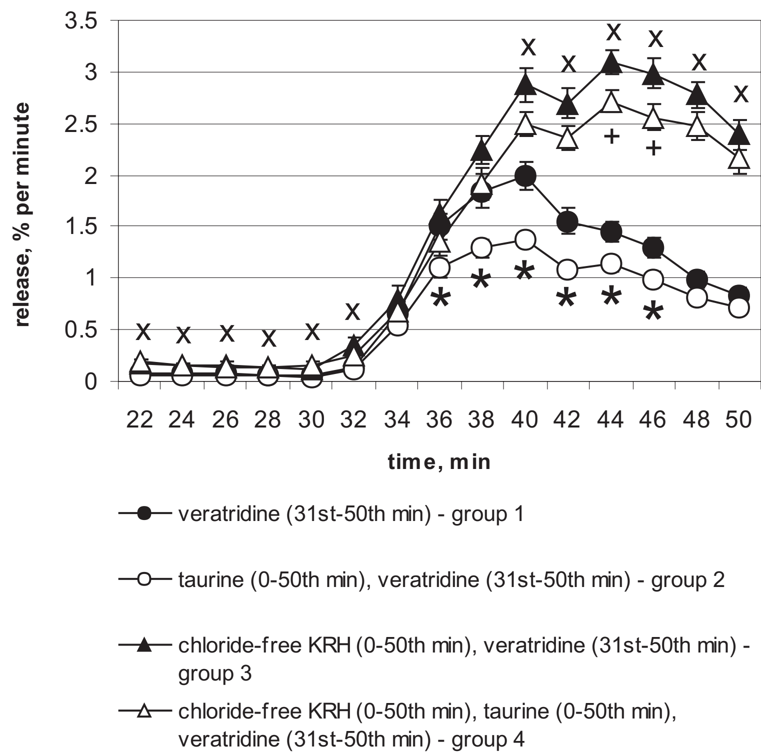

Figure 4A

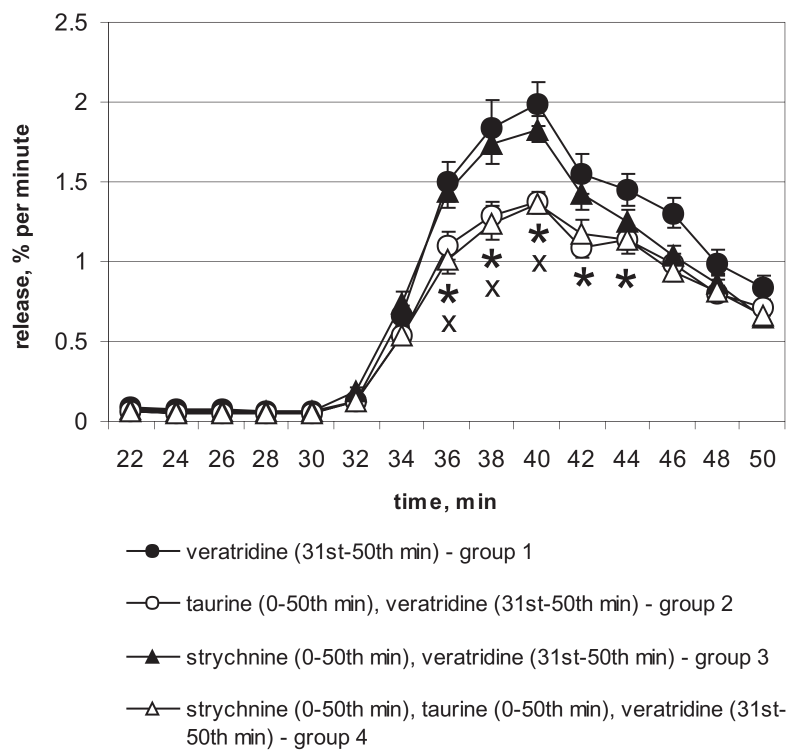

Figure 4B

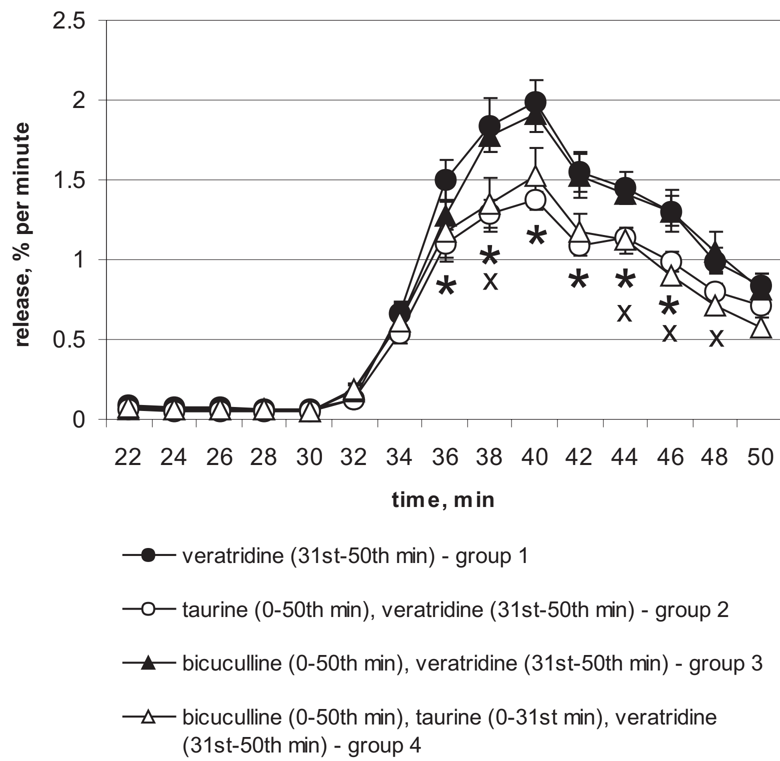

Figure 4C

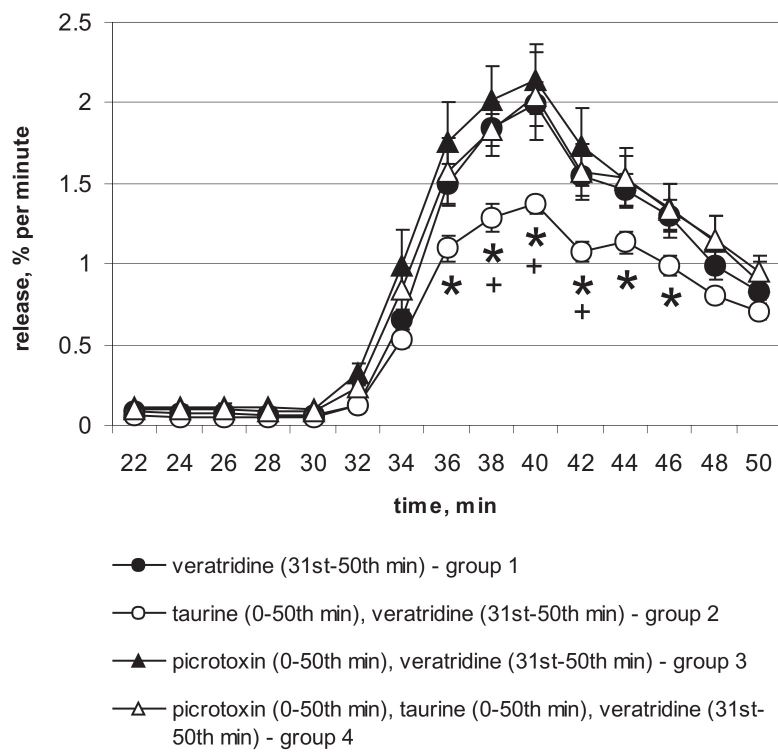

Figure 5A

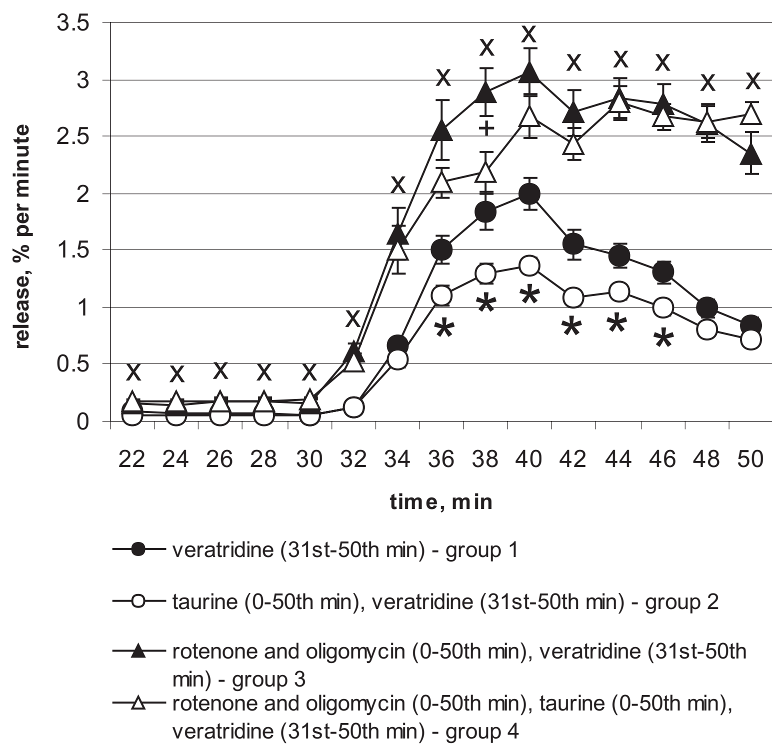

Figure 5B