

Differential effects of nipecotic acid and 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol on extracellular γ -aminobutyrate levels in rat thalamus

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Abstract

Using the microdialysis technique and HPLC (high-performance liquid chromatography) determination of amino acids, the extracellular concentrations of γ -aminobutyrate (GABA), glutamate, aspartate and a number of other amino acids were determined in rat thalamus during infusion through the microdialysis tubing of the GABA transport inhibitors 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (THPO) and nipecotic acid. Administration of 5.0 mM THPO led to a 200% increase in the extracellular GABA concentration. Simultaneous infusion of THPO and GABA (50 μ M) increased the extracellular GABA concentration to 1200% of the basal level whereas GABA alone was found to increase the GABA level to 500%. If nipecotic acid (0.5 mM) was administered together with GABA (50 μ M) the extracellular concentration of GABA was not increased further. While administration of GABA alone or GABA together with nipecotic acid had no effect on the extracellular levels of glutamate and aspartate it was found that GABA plus THPO increased the extracellular concentration of these amino acids. GABA administered alone or together with nipecotic acid or THPO led to relatively small but significant increases in the extracellular concentrations of the amino acids glycine, glutamine, serine and threonine. The results demonstrate that THPO, which preferentially inhibits glial GABA uptake and which is not a substrate for the GABA carriers, was more efficient increasing the extracellular concentration of GABA than nipecotic acid which is a substrate and an inhibitor of both neuronal and glial GABA uptake. This indicates that GABA uptake inhibitors that are not substrates for the carrier and which preferentially inhibit glial GABA uptake may constitute a group of drugs by which the efficacy of GABAergic neurotransmission may be enhanced. © 1997 Elsevier Science B.V.

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1. Introduction

Central inhibitory neurotransmission mediated by γ -aminobutyrate (GABA) is thought to be terminated primarily by uptake of GABA into the presynaptic GABAergic nerve endings or surrounding astroglial cells (Schousboe, 1981, 1990; Krosggaard-Larsen et al., 1987). The uptake of GABA is mediated by high affinity GABA transporters several of which have been recently cloned and characterized (Guastella et al., 1990; Nelson et al., 1990; Lopez-

Corcuera et al., 1992; Borden et al., 1992, 1994, 1995a; Clark et al., 1992). Based on these cloning studies, which have identified at least four distinct carriers, as well as from previous investigations of the pharmacological characteristics of GABA uptake in brain cell preparations including primary cultures of neurons and astroglial cells as well as cell lines, it has repeatedly been suggested that neuronal and astroglial GABA uptake exhibit distinctly different pharmacological properties (Krosggaard-Larsen et al., 1987; Schousboe et al., 1991; Borden et al., 1995b; Borden, 1996). With regard to the maintenance of a steady state level of neurotransmitter GABA it has been pointed out that the relative activity of presynaptic and astroglial GABA uptake processes is of paramount importance as

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only reuptake into the presynaptic endings of the GABAergic neurons allows recycling of neurotransmitter GABA (Schousboe, 1990). As GABA neurotransmission to a large extent is based on reutilization of GABA, a drain on the relevant GABA pool by uptake into astroglial cells and subsequent degradation via transamination and tricarboxylic acid cycle activity would presumably reduce the optimal GABAergic inhibitory tonus (Schousboe, 1990).

Seizure activity and epileptic episodes are associated with aberrations in the equilibrium between excitatory and inhibitory activity (Meldrum, 1975; Heinemann and Jones, 1990). Accordingly, a reduction in the efficacy of GABAergic neurons normally results in seizures or epileptic episodes (Heinemann and Jones, 1990). In recent years it has been established that compounds capable of inhibiting GABA transport and in particular astroglial uptake can act as anticonvulsive agents (Krogsgaard-Larsen et al., 1987, 1994; Schousboe et al., 1991). Examples are tiagabine (Nielsen et al., 1991) as well as (4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol) (THPO) and its related GABA analogues (Schousboe et al., 1991), all of which have structural analogy to nipecotic acid, one of the first recognized potent and specific inhibitors of high affinity GABA uptake (Krogsgaard-Larsen and Johnston, 1975). The ability of nipecotic acid or its derivative *cis*-4-hydroxynipecotic acid to inhibit seizure activity seems to depend on the seizure model under investigation, whereas THPO more consistently has been found to protect experimental animals against seizures induced pharmacologically or by other stimuli (Croucher et al., 1983; Schousboe et al., 1983; Gonsalves et al., 1989). One prominent pharmacological difference between nipecotic acid and THPO is that while the latter compound preferentially inhibits glial GABA uptake, nipecotic acid inhibits neuronal and glial GABA uptake equipotently and more efficiently than THPO (Schousboe et al., 1991). Another important difference between the two inhibitors is that nipecotic acid can be transported by the GABA carriers while THPO cannot (Schousboe and Westergaard, 1995). As the extracellular GABA concentration in the brain may be determined by the efficacy of the high affinity GABA transporters and may be an important factor in the generation of seizures, the present study was undertaken in order to investigate the effect of the two GABA uptake inhibitors on the extracellular level of GABA using the microdialysis technique combined with high performance liquid chromatography (HPLC) based determination of amino acids. This technique has previously been used to investigate the effects of nipecotic acid on the extracellular GABA concentration in different brain areas (Lerma et al., 1984; Kapoor et al., 1990; Campbell et al., 1993) but no previous studies have been performed using THPO. To further investigate whether treatment of rats with THPO and nipecotic acid may affect other amino acids metabolically or functionally linked to GABA, the extracellular levels of glycine (Gly), aspartate (Asp), glutamate (Glu) and glu-

tamine (Gln) were also monitored. Amino acids less closely associated with GABA such as serine (Ser) and threonine (Thr) were also measured extracellularly during the exposure of the animals to the two drugs.

2. Experimental procedures

2.1. Implantation of dialysis probes

Dialysis probes were prepared as described earlier in detail (Juhász et al., 1989). Briefly, hollow fibers of 5000 Da molecular mass cut-off were placed into the tip of a stainless steel tube (27 Gauge). The outside diameter of the fibers was 0.2 mm, the length of the surface was 3 mm. Glass capillaries pulled from Corning borosilicate glass tubes served as inlet and outlet of the probe forming a concentric type dialysis sampler. On the basis of electrochemical determination of efflux of compounds from the probe, recovery of GABA and THPO was estimated to 10%. Polyethylene connecting tubes attached the probe to the dialysis pump (KD Scientific Dialysis Pump, Stoelting, Denmark). Artificial cerebrospinal fluid (ACSF) containing 140 mmol Na⁺, 3 mmol K⁺, 1.2 mmol Ca²⁺, 2 mmol Mg²⁺ and 144 mmol Cl⁻ was used for perfusion. ACSF was osmotically equilibrated with NaCl and pH was adjusted to 7.2. The amino acid content of the ACSF was checked measuring an ACSF sample with HPLC before use and ACSF was used only if it gave rise to a straight line (with background noise) at the detector sensitivity applied for the *in vivo* sample analysis. To keep the perfusion solution amino acid free, sterile, disposable 1 ml syringes were used in the dialysis pump. The inlet and outlet tubes were cleaned with hydrogen peroxide to prevent amino acid contamination.

Wistar rats of 300 g body weight were anaesthetized with 1% Halothane/air mixture and placed into a stereotaxic head holder. Rectal temperature was measured and controlled by a heating lamp. Opening the skull, two dialysis probes were implanted into the ventrolateral thalamus on both sides (AP = -2, L = 2.5, V = -7 mm on the basis of stereotaxic atlas of Pellegrino and Cushman, 1967). The probes were slowly lowered reaching the final position within 40 min to induce minimal damage in the brain tissue. Perfusion of the probes was performed at a flow rate of 1 µl/min and 30 min samples were collected in 500 µl vials. The samples from the first 1.5 h were not used because of the rapid changes in amino acid concentrations right after the implantation. In order to estimate a possible lag-period when the dialysis buffers were changed, colored solutions were used. The lag-period was estimated to be a few minutes and thus can be considered negligible using 30 min collection periods. Rats were separated into 4 groups. In group 1, 50 µM GABA was added to the ACSF solution after 3 control samples. In group 2, 5.0 mM THPO was applied in the perfusate after collection of 3

control samples. In group 3, 50 μ M GABA was added to the ACSF for 1.5 h then 5.0 mM THPO was added to it. In group 4, the same protocol as that in group 3 was used but 0.5 mM nipecotic acid was added instead of THPO. Under these conditions it is estimated that the concentration of drugs around the dialysis probe is approximately 10% of the concentration in the dialysis buffer (Juhász et al., 1989), i.e. for both drugs close to the respective K_i values as inhibitors of GABA uptake (Schousboe and Westergaard, 1995).

2.2. Amino acid analysis and data processing

Amino acid analysis was performed on a Pharmacia LKB AminoSys High Performance Liquid Chromatography (HPLC) system. The *ortho*-phthalaldehyde (OPA) derivatization method (Lindroth and Mopper, 1979) was used to measure amino acids at high sensitivity. Using HP Hypersil ODS 2.1 \times 200 mm bore columns with a 20 mm precolumn, all amino acids in question could be separated. Eluent A was 0.1 M phosphate buffer, 0.4 v/v% THF (tetrahydrofurane) and 0.02 v/v% TEA (tetraethylene ammonium), pH 7.2; eluent B was 70% acetonitrile in eluent A adjusted to pH 7.2 with phosphoric acid. OPA derivatives of amino acids were detected with a fluorescence detector using 305–395 nm excitation and 430–470 nm emission filters. Sensitivity of the method was in the low picomolar range. Automatic evaluation of the chromatograms was performed on-line by PE NELSON software (Perkin Elmer). Peak areas were calculated and statistically analyzed by ANOVA. Because of the relatively large dispersion of permeability of dialysis probes, an experimental protocol was used calculating all data in percent of the control values.

3. Results

The effect of THPO and nipecotic acid on the extracellular GABA concentration was investigated in a set of experiments where the two drugs were administered through the microdialysis probe contralateral to the probe in the control side. The baseline (control) levels (μ M) of amino acids as measured in the dialysates were: Glu 6.6 ± 0.3 ; Asp 1.7 ± 0.03 ; Gly 8.7 ± 0.6 ; GABA 0.7 ± 0.2 ; Thr 4.2 ± 0.7 ; Ser 10.3 ± 1.5 (all values are averages \pm SEM, $n = 22$). Fig. 1 shows the time-course of the increase in the GABA concentration observed after administration of 5.0 mM THPO through the microdialysis probe. It is seen that during 30–60 min, the extracellular GABA concentration increased to about 200% of that in the control side and that it remained elevated at this level during the remaining dialysis period (3 h). The GABA level on the control side was constant during this period. Fig. 2 demonstrates a differential action of THPO and nipecotic acid on the steady state increase in the extracellular

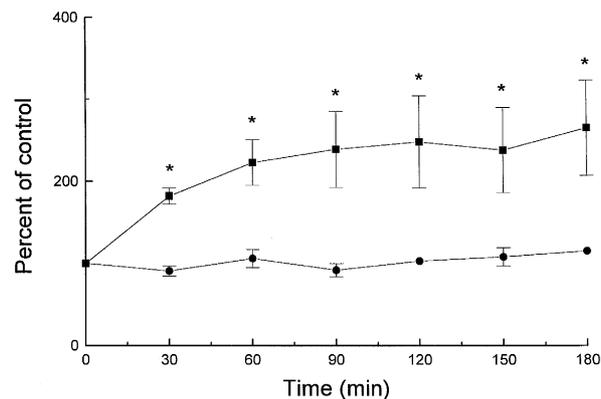


Fig. 1. Time-course of the increase in the extracellular GABA concentration (% of control) in thalamus of rats implanted with microdialysis probes as described in Section 2. Dialysis probes in the control side were perfused with ACSF and in the experimental, contralateral side with ACSF containing 5.0 mM THPO. Control values are shown by circles and values from the THPO treated side are indicated by squares. Results are averages of 6 animals with S.E.M. values shown by vertical bars. The asterisks indicate statistically significant differences from the controls ($P < 0.01$, Analysis of variance (ANOVA)).

lar GABA concentration produced by infusion of GABA (50 μ M) through the dialysis fiber. It is seen that infusion of GABA led to a 500% increase in extracellular GABA during the time period 30–90 min and that administration of nipecotic acid (0.5 mM) together with GABA had no further effect on the extracellular GABA level during the following 90 min. However, simultaneous infusion of 5.0 mM THPO further increased the GABA concentration reaching a level of 1200% 90 min after the start of the infusion of THPO together with GABA. In order to rule

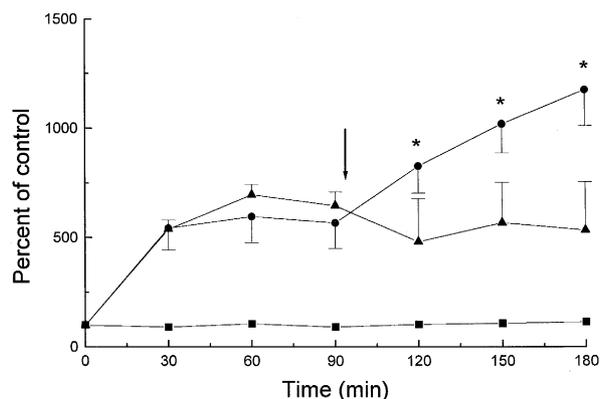


Fig. 2. Time-course of the increase in the extracellular GABA concentration (% of control) in thalamus of rats implanted with microdialysis probes as described in Section 2. The probe in the control side (\blacksquare) was perfused with ACSF and that in the contralateral, experimental side (\bullet or \blacktriangle) with 50 μ M GABA (0–100 min) and subsequently (at arrow) with 50 μ M GABA and 5.0 mM THPO (\bullet) or 50 μ M GABA and 0.5 mM nipecotic acid (\blacktriangle). Results are averages of 5 experimental animals with S.E.M. values shown by vertical bars. Asterisks indicate statistically significant differences ($P < 0.01$, ANOVA) from the extracellular GABA concentration in the respective group of animals prior to the addition of THPO or nipecotic acid.

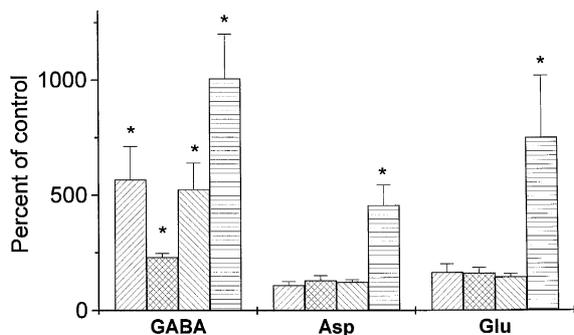


Fig. 3. Increase in the extracellular concentration (% of control) of GABA, aspartate (Asp), or glutamate (Glu) in thalamus of rats implanted with microdialysis probes as described in Section 2. The values represent the averages \pm S.E.M. (vertical lines) of 5 experimental animals of the amino acid concentrations measured after 180 min (see Fig. 2) of perfusion with 50 μ M GABA (diagonally striped to the right), 5.0 mM THPO (hatched), 50 μ M GABA plus 0.5 mM nipecotic acid (diagonally striped to the left) and 50 μ M GABA plus 5.0 mM THPO (horizontally striped). Asterisks indicate statistically significant differences from the amino acid levels in the control side ($P < 0.01$, ANOVA).

out the possibility that other factors than differences in potency as uptake inhibitors between nipecotic acid and THPO may influence their ability to affect the extracellular GABA concentrations, a set of experiments was performed using a nipecotic acid concentration of 5.0 mM in the presence of 50 μ M GABA. Even at this high concentration, nipecotic acid did not increase the extracellular GABA concentration above the level achieved by administration of GABA alone (results not shown).

Since the possibility exists that changes in the extracel-

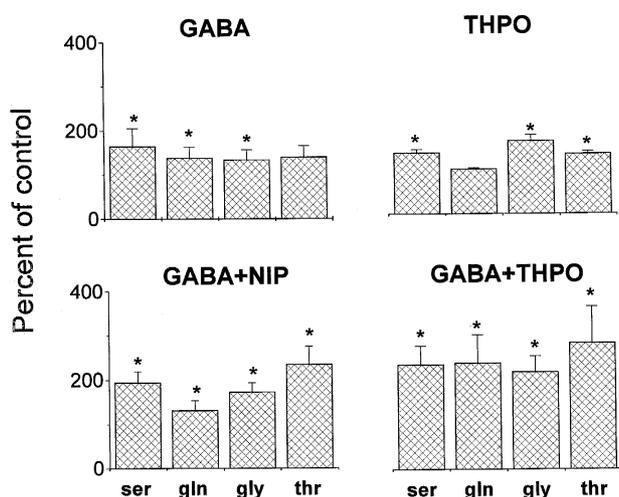


Fig. 4. Increase in the extracellular concentration (% of control) of serine (Ser), glutamine (Gln), glycine (Gly) and threonine (Thr) in thalamus of rats implanted with microdialysis probes as described in Section 2. The values represent the averages \pm S.E.M. (vertical lines) of 5 experimental animals of the amino acid concentrations measured after 180 min (see Fig. 2) of perfusion with 50 μ M GABA, 5.0 mM THPO, 50 μ M GABA plus 0.5 mM nipecotic acid (NIP) or 50 μ M GABA plus 5.0 mM THPO. Asterisks indicate statistically significant differences from the amino acid levels in the control side ($P < 0.01$, ANOVA).

lular level of GABA may affect the homeostatic mechanisms responsible for maintenance of the extracellular levels of other amino acids, the concentrations of a series of other amino acids were also determined. Fig. 3 shows that while the extracellular GABA level was elevated by infusion of GABA or THPO alone as well as by nipecotic acid or THPO in combination with GABA, the concentrations of aspartate and glutamate were only elevated after infusion of GABA together with THPO, a condition leading to an extremely high (1000%) increase in the extracellular GABA concentration. The increase in extracellular GABA (250–500%) seen during infusion of GABA, THPO or GABA plus nipecotic acid did not affect the levels of Asp and Glu. As seen in Fig. 4, the levels of Gly, Gln, Ser and Thr were elevated when GABA was infused together with either nipecotic acid or THPO, the effect being most pronounced in the latter case, i.e. when GABA and THPO were administered together. When GABA or THPO was administered separately, the extracellular levels of the 4 amino acids mentioned above were less increased and in some cases the increases did not reach the level of significance ($P < 0.01$).

4. Discussion

In relation to previous observations that intracerebral administration of THPO can prevent seizures in rats or mice or induce sleep in cats (Croucher et al., 1983; Wood et al., 1983; Gonsalves et al., 1989; Juhász et al., 1991), it is interesting that infusion of THPO through the microdialysis probe led to a considerable increase in the extracellular concentration of GABA. The anticonvulsive and sleep promoting actions of THPO may thus be correlated with the increased GABA level which is likely to exert a tonic inhibition of neuronal activity. It is noteworthy that the increase in extracellular GABA induced by administration of GABA and thus inferring an overload of the GABA transporters was even more pronounced when THPO was administered together with GABA, indicating a pronounced functional inhibition of the GABA carriers. As the astrocytic carrier appears to be more sensitive than the neuronal counterpart to inhibition by THPO (Schousboe et al., 1991) this may indicate that when the carriers are working at maximal activity, the glial cells may be important for removal of GABA. This ability of THPO to elevate the extracellular GABA concentration has also been demonstrated in mixed cultures of GABAergic neurons and astrocytes depolarized by 50 mM K^+ in order to enhance neuronal GABA release (Westergaard et al., 1992). It was surprising that in spite of the fact that nipecotic acid is more potent than THPO as an inhibitor of GABA uptake (Schousboe et al., 1979, 1981), it was found to be unable to increase the extracellular GABA concentration above the level induced by infusion of GABA alone through the dialysis probe. It should be kept in mind, however, that

while THPO cannot itself be transported, nipecotic acid is a substrate for the GABA carrier (Schousboe and Westergaard, 1995). The inability of nipecotic acid to further increase the extracellular GABA concentration when administered together with 50 μ M GABA was also unexpected in the light of previous findings that nipecotic acid when given alone could elevate the extracellular GABA concentration in hippocampus, caudate-putamen and the ventrolateral medulla (Lerma et al., 1984; Kapoor et al., 1990; Campbell et al., 1993). It may be argued that the thalamus may be different from these other brain areas with regard to actions of nipecotic acid but this is unlikely since nipecotic acid is an inhibitor of all known GABA carriers (Borden, 1996). The difference in the action of the two drugs may, however, explain why in some animal models of seizures, nipecotic acid was found to be less active than THPO as an anticonvulsant agent (Gonsalves et al., 1989). This finding also underlines the notion (Schousboe et al., 1983) that selective inhibition of astrocytic GABA uptake is more efficient than inhibition of both neuronal and glial uptake in producing an anticonvulsant action. This may indicate that the neuronal carriers which would be completely blocked in the presence of nipecotic acid but only partly so in the presence of THPO, play a functional role in releasing GABA under these experimental conditions. In this context it should be noted that when THPO was infused together with GABA, the extracellular levels of the two excitatory amino acids aspartate and glutamate were also significantly elevated. This may be important for the understanding of the increased GABA level since release of GABA induced by glutamate in certain neurons involves preferentially the cytoplasmic, non-vesicular GABA pool from which GABA is released by reversal of the carrier (Belhage et al., 1993).

The mechanism responsible for the increased extracellular levels of glutamate, aspartate and a number of other amino acids when the extracellular GABA was increased is not easily understood. Under conditions where the inhibitory tonus of the tissue would be expected to be increased, i.e. in the presence of increased extracellular GABA levels, the release of the excitatory amino acid neurotransmitter glutamate and aspartate would be expected to decrease due to the inhibitory action of GABA (Meier et al., 1984; Kardos et al., 1994). The possibility does, however, exist that GABA disinhibition may provoke an enhanced release of these amino acids (Nielsen et al., 1989; Krogsgaard-Larsen et al., 1994; Kardos et al., 1994). An increased extracellular level of glutamate and aspartate may, in turn, lead to release of other amino acids due to depolarization and reversal of carriers and may thus contribute to the relatively modest increase in the extracellular levels of the other amino acids investigated. It may be argued that this effect on the levels of glycine, serine, threonine and glutamine may be exerted by THPO itself and not indirectly via its ability to increase GABA levels. However, since the effect was almost the same as that seen

when GABA levels were elevated by GABA itself or by GABA in the presence of nipecotic acid, a direct action of THPO is unlikely. Moreover, the effect of THPO alone was less pronounced than that of THPO plus GABA. This is also the case for aspartate and glutamate as the extracellular levels of these two amino acids were only elevated when GABA and THPO were administered together, a condition leading to an excessively high extracellular GABA concentration. The increased extracellular levels of aspartate and glutamate observed when THPO was co-administered with GABA are approaching those seen under conditions, e.g. ischemia leading to excitotoxic neuronal damage (Benveniste et al., 1984). No attempt was, however, made in the present study to evaluate the rat brains for possible neuronal damage.

The results clearly indicate that GABA uptake inhibitors that are not substrates for the carrier and which preferentially inhibit glial GABA uptake may constitute an interesting group of drugs by which the efficacy of GABAergic neurotransmission may be enhanced leading to higher thresholds for seizure activity. In analogy to what has been shown for tiagabine, compounds with a profile of action similar to that of THPO may have anticonvulsant properties in animal models and may show antiepileptic effects.

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