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COMPARISON OF RESULTS OBTAINED WITH DIFFERENT METHODS FOR ESTIMATING GABA TURNOVER IN RAT NEOSTRIATUM

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Abstract—Different methods for measuring GABA turnover in rat brain were compared.

One method was based on the irreversible inhibition of GABA transaminase (EC 2.6.1.19) by microinjection of γ-vinyl-GABA into neostriatum of rat. The accumulation of GABA was almost linear for 4 hr. The GABA turnover in control animals was estimated to be 25.8 ± 1.1 nmole/mg protein/hr. Another method was based on the post mortal increase in GABA level in an 8 min interval after decapitation. This method gave a GABA turnover of 54.3 ± 4.8 nmole/mg protein/hr in neostriatum.

The methods were compared with respect to their ability to detect the effect of high doses of diazepam and morphine on the turnover rate of GABA. The GABA transaminase inhibition method resulted in a 27% and a 17% decrease in GABA turnover for diazepam and morphine respectively. The *post mortem* method did not detect any change in GABA turnover after administration of these drugs.

Hypoglycemia leads to a decrease in GABA turnover of 17% with the GABA transaminase inhibition method and a 43% decrease in GABA turnover with the *post mortem* method.

The advantages and limitations of the methods for estimating GABA turnover changes during drug exposure is discussed, and are compared with results from a third method based on steady state kinetics extracted from the literature.

The effect of drugs on GABAergic† neurotransmission is most often given as a change in the GABA level. This change, however, could be the result of different mechanisms. Both an inhibition of metabolism of GABA and a decreased utilization of GABA may give an increase in GABA level. A more sensitive way of detecting dynamic or reversible changes in transmitter function is to measure the turnover of the transmitter.

Until now three principally different methods for GABA turnover studies have been suggested (for a review, see [1]). These are: (1) labelled precursor infusion; (2) inhibition of GABA transaminase, the major GABA degrading enzyme; (3) measurement of the *post mortem* GABA accumulation.

For acetylcholine and biogenic amines, methods have been developed for turnover studies based on labelled precursors [2, 3]. These models, using glucose as a precursor, have also been applied on GABA turnover [4, 5]. The use of [^{13}C]-labelled glucose for GABA turnover estimations during drug exposure has been suggested [4]. Although there is a clear disadvantage in that glucose is separated by several steps from GABA production, this method has been extensively used to study the effect of drugs on GABA turnover.

The use of GABA-transaminase inhibitors takes advantage of the fact that GABA is almost completely metabolized by GABA-transaminase (GABA-2-oxoglutarate aminotransferase, EC 2.6.1.19). It follows that the initial GABA accumulation is an estimate for GABA synthesis or turnover rate. Several GABA-transaminase inhibitors have been introduced to measure the effect of drugs on GABA turnover [6]. They differ greatly in their specificity and efficacy (for a review, see [1]).

The *post mortem* GABA accumulation method is based on the fact that GABA catabolism is nearly completely inhibited because of anoxia after death, due to depletion of 2-oxoglutarate and NAD [7]. This approach was used as the first attempt to calculate GABA turnover [7], and it has been claimed that this method has been used successfully to show that GABA is involved in the feedback action of estradiol on prolactin and gonadotropin release [8].

The object of the present investigation was to compare results from the two last methods for measuring GABA turnover with results from the literature on steady state kinetics. Three drugs with different modes of action on the GABAergic neurons were selected: diazepam, morphine and insulin.

Diazepam and morphine were selected because they have previously been shown to give large decreases in GABA turnover when applying the steady state kinetics [9, 5]. Diazepam interacts with the GABA-receptor ionophore complex. Potentiation of postsynaptic GABAergic transmission by benzodiazepines is thought to lead to downregulation of GABA turnover [9]. Morphine is suggested to interact with striatal opioid receptors, which are modulating the GABAergic interneurons in striatum [5]. However, the doses in our study are not chosen to evaluate the therapeutic mechanism of action, but rather as tools for studying changes in GABA turnover. Doses selected are similar to earlier

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† Abbreviations used: GABA, γ-amino butyric acid; GVG, gamma-vinyl-GABA; GAD, glutamic acid decarboxylase.

reported studies to allow comparison. Insulin was selected because it induces hypoglycemia and thereby interacts in a metabolic manner with the GABAergic transmission. Insulin lowers the brain glutamate level due to its hypoglycemic action [10]. The decreased level of the immediate precursor for GABA synthesis could be the mechanism of action of this drug on GABA metabolism. Data for steady state kinetics for hypoglycemia were not available, since it would interact with the precursor glucose.

This study was limited to neostriatum, where both GABAergic cell bodies and GABAergic terminals are present [11].

MATERIALS AND METHODS

Chemicals. D,L-α-Aminoadipic acid was obtained from Sigma (St Louis, MO), and *o*-phthaldialdehyde from E. M. Merck AG (Darmstadt, F.R.G.) γ-Vinyl-GABA (GVG) was a gift from Merrel Int. Res. Center (Strasbourg, France). Valium inj. 5 mg diazepam/ml was obtained from Hoffman La Roche (Switzerland), Hypnorm (10 mg fluanisone and 0.2 mg fentanyl/ml) from Leo (Helsingborg, Sweden), morphine inj, 10 mg/ml from NAF-laboratoriene A/S (Norway) and Insulin Actrapid from Novo Industries A/S (København, Denmark).

Animals. Male Wistar rats weighing 150–250 g obtained from Møllegard-Hansens Avlslaboratorium (Vejby, Denmark) were used throughout the study. The animals were grouped and held at 24° in a 12 hr light–dark cycle, receiving food and water *ad libitum*. After GVG injection the rats were caged singly. All rats were killed by decapitation. Rats receiving insulin were denied access to food but were given water *ad libitum* for 24 hr prior to the experiment.

Inhibition of GABA transaminase. GVG, dissolved in 0.9% NaCl to 20 mg/ml, was microinjected stereotaxically into the neostriatum under light ether anesthesia in order to allow rapid recovery of the animals, using a David Kopf Stereotaxical apparatus. One microlitre was injected continuously with a rate of 0.25 μl/min via a stainless steel cannula (25 gauge) connected to a 25 μl syringe driven by a Palmer slowly injecting pump. The coordinates were: anterior–posterior with respect to bregma + 1.0 mm, lateral with respect to the midline suture −3.0 mm, dorso–ventral with respect to dura −5.0 mm. Control rats were injected with an equal volume 0.9% NaCl. Valium (10 mg diazepam/kg i.p.), morphine (20 or 40 mg/kg s.c.) or 0.9% NaCl (s.c.) was injected 30 min prior to the GVG injection, insulin (140 IU/kg i.p.) 60 min prior.

To prepare the animals for the injection a hole was drilled in the skull down to dura under Valium-Hypnorm anesthesia (2.5 mg diazepam/kg i.p. and Hypnorm 0.5 ml/kg s.c.). The animals were allowed to recover for at least 48 hr before injection in order to get rid of possible effects of the anesthetics on GABA turnover. It is not found residual radioactivity in rat brain 48 hr after injection of 0.6 mg/kg tritiated diazepam [12].

Post mortem studies. Rat brains for the *post mortem* studies were excised and incubated in 0.9% NaCl in a thermostated water bath maintained at 37°. One brain half was incubated for 2 min after decapitation and the other for 10 min. Insulin-treated rats were used after righting reflex had disappeared. Morphine or diazepam treated rats were used 1 hr after drug administration or when catalepsy or sedation respectively was clearly evident.

Tissue preparation. The rat heads were cooled in liquid N_2 for 10 sec after decapitation or after incubation to reduce brain temperature to near freezing point. A slice (5–10 mg) of neostriatum was dissected out and homogenized in 1 ml ice-cold 2.5% trichloroacetic acid containing 40 μM α-aminoadipic acid as internal standard. Samples were extracted for at least 20 min on ice, and subsequently centrifuged for 20 min at 20,000 *g* in a Sorwall RC-2 centrifuge.

Protein determination. The pellets were dissolved in 0.5 M NaOH containing 0.2% sodium dodecyl sulfate and used for protein determination by the method of Lowry *et al.* [13].

Amino acid measurements. The supernatant from the centrifugation was extracted four times with an equal volume of water saturated ether, and used for amino acid analyses on HPLC after precolumn fluorescence derivatization with *o*-phthaldialdehyde [14, 15]. The HPLC was a Varian Model 5000 LC with Supelco LC 18 Column (particle size 5μ, length 25 cm, i.d. 4.6. mm). Mobile phases were A, 50 mM sodium phosphate buffer pH 5.25 and B, methanol HPLC grade (Rathburn), gradient from 25 to 75% B in 20 min. Detector: Kratos Fs 970 LC fluorometer, excitation wavelength 330 nm, emission filter 418 nm. The data for GABA and glutamate content were based on peak areas, computed by a Varian Vista 401 data system.

GABA transaminase activity measurement. Tissue for measurement of GABA transaminase activity was homogenized in 0.32 M sucrose. The enzyme activity was measured according to [16].

Statistics. Statistical analyses were performed by Wilcoxon's two sample test.

RESULTS

The inhibition of GABA transaminase for 2 and 4 hr with 20 μg GVG intrastriatal was used as a method for measuring the effect of diazepam, morphine and insulin on GABA turnover. The GABA transaminase activity measurements showed a decrease in enzyme activity of 87% after GVG treatment in the injected striatum after 4 hr, and no inhibition on the contralateral side (Table 1). Thus the contralateral side was selected as a zero time control. Earlier work in our laboratory has shown that the selected dose was the lowest dose giving maximal inhibition of GABA transaminase.

The GABA accumulation was nearly linear for 4 hr (Fig. 1). GABA turnover was estimated to be 25.8 ± 1.1 nmole/mg protein/hr for the control group (0.5 ml 0.9% NaCl s.c.) in the time interval 0–2 hr after GVG administration (Table 2). Diazepam treatment (10 mg diazepam/kg i.p.) resulted in sedation. The GABA turnover decreased to 18.7 ± 1.9 nmole/mg protein/hr in the time interval 0–2 hr after GVG administration, which corresponds to a 27% decrease compared to normal. Morphine treatment (20 or 40 mg morphine/kg s.c.), resulted

Table 1. GABA-transaminase activities after inhibition of GABA-transaminase in striatum with 20 μg GVG for 4 hr *in vivo*

	GABA-transaminase activity
Saline (1 μl 0.9% NaCl)	240 ± 27 (8)
GVG contralateral	260 ± 25 (7)
GVG (1 μl 20 mg/ml GVG)	32 ± 5 (7)**

GABA-transaminase activities given as nmole/mg protein/hr.
Mean values ± SEM, number of animals in parentheses.
**P < 0.01.

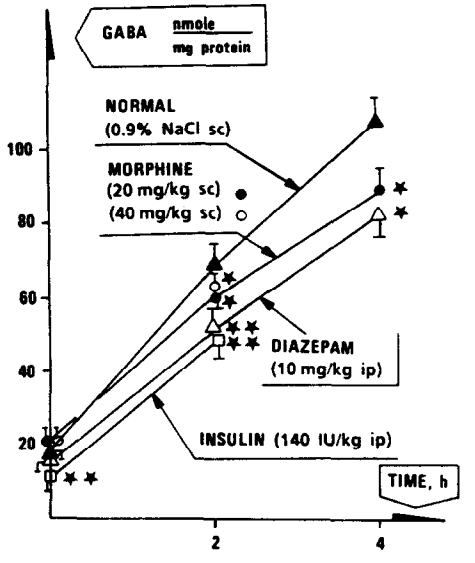

Fig. 1. GABA levels given as nmole/mg protein in rat neostriatum after *in vivo* GABA-transaminase inhibition for 2 or 4 hr with 20 μg GVG of rats treated with saline i.p. diazepam (10 mg/kg i.p.), morphine (20 or 40 mg/kg s.c.) or insulin (140 IU/kg i.p.). Mean values ± SEM of at least 5 animals. *P < 0.05, **P < 0.01.

in catalepsy and a decrease in GVG induced GABA accumulation of 17%, to a turnover rate of 21.4 ± 1.4 nmole/mg protein/hr after 20 mg/kg s.c. in the time interval 0–2 hr after GVG administration. No dose dependency for morphine treatment was observed in this study. The effect of diazepam and morphine on GVG induced GABA accumulation sustained for at least 4 hr (See Table 2). Insulin-induced hypoglycemia (140 IE insulin/kg i.p.) resulted in a decrease in GABA turnover of 17%. In the case of insulin treated rats, the final glutamate level was 43 ± 5.2% (SEM) of control in untreated neostriata.

Results from the *post mortem* method are shown in Table 3. When rats were given saline i.p., the turnover was estimated to be 54.3 nmole/mg protein/hr in the period 2–10 min *post mortem*. Neither morphine (20 mg/kg s.c.) nor diazepam (10 mg/kg i.p.) changed the rate of *post mortem* GABA accumulation even with a diazepam dose twice that given during GABA-transaminase inhibition. In the case of hypoglycemic rats (140 IU insulin/kg), the turnover rate of GABA was estimated to be 31.0 nmole/mg protein/hour, corresponding to a 43% decrease in GABA turnover rate compared to control. In this case the glutamate level was 51 ± 7% of control level.

DISCUSSION

The present study compares different methods for estimating GABA turnover rate *in vivo*. All methods reported so far measure the rate of GABA metabolism and not strictly the rate of transmitter release. Our results within inhibition of GABA-transaminase and the results from the literature with use of labelled precursors gave qualitatively the same results when comparing the effect of drugs, whereas the post mortal GABA increase only detected changes in GABA formation when the level of the precursor was reduced.

Several conditions should be fulfilled for use of GABA-transaminase inhibitors as tools for estimating GABA turnover. The inhibition should be specific, rapid and stable. The GABA-transaminase

Table 2. The effect of diazepam, morphine and insulin on GVG induced GABA accumulation

Drug treatment	Time interval (hr)	GABA turnover
Saline (0.5 ml s.c.) [11]	2	25.8 ± 1.1
Diazepam (10 mg/kg i.p.) [8]	2	18.7 ± 1.9**
Morphine (20 mg/kg s.c.) [9]	2	21.4 ± 1.4*
Morphine (40 mg/kg s.c.) [5]	2	21.8 ± 1.2*
Insulin (140 IU/kg i.p.) [8]	2	20.5 ± 1.4**
Saline (0.5 ml s.c.) [6]	4	23.2 ± 1.2
Diazepam (10 mg/kg i.p.) [6]	4	17.2 ± 1.3*
Morphine (20 mg/kg s.c.) [6]	4	18.8 ± 1.4*

GABA-transaminase inhibition for 2 and 4 hours with 20 μg GVG intrastriatal as a method for estimating GABA turnover. GABA turnover given as nmole/mg protein/hr. Mean values ± SEM, number of animals in parentheses.
* P < 0.05. ** P < 0.01.

Table 3. The effect of diazepam, morphine and insulin on GABA accumulation *post mortem*

	GABA 2 min	GABA 10 min	GABA TR	% of normal
Normal saline i.p. [7]	17.1 ± 1.2	24.3 ± 1.1	54.3 ± 4.8	100
Diazepam 10 mg/kg i.p. [8]	18.2 ± 1.2	25.7 ± 0.8	55.9 ± 6.5	103 ± 12
Insulin 140 IE/kg i.p. [6]	11.9 ± 1.4*	16.1 ± 2.1*	31.0 ± 6.7*	57 ± 12*
Morphine 20 mg/kg i.p. [7]	20.1 ± 1.5	26.5 ± 2.1	48.1 ± 14.6	89 ± 27

GABA accumulation in the time interval 2–10 min *post mortem* as a method for estimating GABA turnover.

GABA turnover (GABA TR) given as nmoles/mg protein/hr. Mean values ± SEM, number of animals in parentheses.

*$P < 0.05$.

GABA level given as nmole/mg protein.

inhibitors are grouped in pyridoxal phosphate-interacting and substrate analogue types [6]. The first group would interact with several pyridoxal phosphate-requiring enzymes, including GABA-transaminase and GAD (glutamic acid decarboxylase (EC 4.1.1.15)) and are therefore not sufficiently specific. Even so, the GABA-transaminase inhibitor aminooxyacetic acid [17], which is interacting with pyridoxal phosphate, has been extensively used for the determination of GABA turnover [18, 19]. The other group of GABA transaminase inhibitors makes use of substrate analogues. γ-Vinyl-GABA (GVG) and gabaculine seem to be the most suitable representatives in this group [1]. Both are highly specific for GABA-transaminase, and interfere little with GAD [6, 20]. GVG has a rapid onset of action and the effect is stable for several hours [21]. Locally applied GABA-transaminase inhibitors for measuring GABA turnover in discrete brain areas has been suggested [22, 23]. By placing the inhibitor locally in the brain, one can avoid some general effects such as sedation, depending on the brain area selected [22]. By operating in the linear phase of GABA accumulation the problem with possible product inhibition of GAD is circumvented. We have shown that the GABA accumulation is nearly linear for 4 hr after GVG administration. It has been shown for gabaculine administered intrastriatally that the GABA accumulation is linear for at least 1 hr, and that the initial one quarter of an hour accumulation rate is equivalent with the later, making measurements over longer time intervals reliable [23].

In support for the use of GABA-transaminase inhibitors as a method for measuring GABA turnover, it is shown that 6–8 days after surgical hemitranssection, GABA turnover decreased in substantia nigra [22].

The normal GABA turnover value found in our study, corresponds closely to earlier reported estimates for rat neostriatum (25 nmole/mg protein/hr) estimated by use of microinjected gabaculine into rat neostriatum [23].

For the *post mortem* method to be suitable, the GABA synthesis and its regulation *post mortem* should reflect the synthesis *in vivo*, and the GABA catabolism should be completely blocked *post mortem*. The GABA accumulation is not linear *post mortem*, due to the compartmentation of amino acids in brain [24]. During the post mortal state, GABA formation changes from one metabolically active glutamate compartment to a less active one [24]. There are two factors which limit the usefulness of this method. To simulate the conditions *in vivo*, one should select short incubation times. However, this fact introduces experimental difficulties because a small increase in GABA will give a low degree of reproducibility. Further, a *post mortem* activation of GAD has been suggested, due to decreased ATP level [25]. From this argument it follows that the estimate for GABA turnover may be too high. It is also shown, however, that GABA catabolism is not immediately or completely inhibited *post mortem* [26]. The basal GABA turnover value in neostriatum was estimated to be in the same order compared to the value obtained with GABA-transaminase inhibition.

The technique applying labelled precursors is not easily applicable for GABA turnover studies. In support of the method, however, is an increase in GABA turnover in substantia nigra following stimulation of the striatonigral tract [27]. The model requires that the immediate precursor for GABA synthesis is used. Glutamate does not penetrate the blood–brain barrier, so glucose has been selected instead. This model requires that GABA is produced from an open compartment with rapid equilibrium with its precursors. This is clearly not fulfilled for GABA metabolism [4]. Glucose does not exclusively label the glutamate pool available for GABA synthesis. Only a fraction of the total glutamate pool is available for GABA synthesis [24]. Further, the kinetic model requires an end product, but the products in GABA catabolism are assimilated into Krebs' cycle and reused. Both a change in specific activity of glutamate and GABA will give a change in GABA turnover, unless one restricts the model to studies with a constant glutamate labelling rate only. The labelling pattern is different in different brain areas, and it has been suggested that the use of this method should be restricted to GABA rich regions [1]. With this method one has arrived at a basal turnover rate even greater than the glucose metabolism in brain [4]. This is due to the fact that only a limited fraction of the total glutamate pool is available for GABA synthesis. Labelled glucose as a precursor for GABA synthesis gave a basal turnover rate in neostriatum of 456 nmole/mg protein/hr [9] or 690 nmole/mg protein/hr [5] (see Table 4).

Table 4. Application of steady state kinetics to estimate the effect of diazepam and morphine on GABA turnover (Extracted from the literature)

Drug treatment	Glu level	GABA level	kGABA	GABA TR
Saline	121 ± 5.1	19 ± 0.9	24 ± 2.6	456 ± 54‡
Diazepam (1 mg/kg i.p.)	—	—	8.43 ± 0.63**	—‡
Saline	110 ± 9.2	23 ± 2.0	34 ± 4.8	690 ± 100†
Morphine (20 mg/kg s.c.)	100 ± 9.2	24 ± 2.0	11 ± 1.8	240 ± 45**†

GABA turnover (GABA TR) in nucleus caudatus of rat. Glutamate and GABA levels given as nmole/mg protein; rate constant for GABA efflux (kGABA) given as hr^{-1}; GABA TR given as nmole/mg protein/hr, obtained by multiplying kGABA with the GABA concentration.
Mean ± SEM for at least 5 animals.
** $P < 0.01$.
Results obtained from ‡ [9], † [5].

In order to evaluate the usefulness of the different methods for estimating changes in GABA turnover during drug exposure, high doses of diazepam, morphine or insulin were administered.

The method applying GABA-transaminase inhibitors will underestimate the real GABA turnover values, since it is not possible to inhibit GABA-transaminase completely by use of GABA-transaminase inhibitors in an acceptable dose. In our study the GABA-transaminase was inhibited by 87%. This dose will not alter GAD activity [22]. Therefore, this method is most suitable for comparative studies such as evaluating the effects of drugs on GABA turnover.

We found a decrease in GVG induced GABA accumulation of 27% after diazepam treatment, which is similar to findings of other authors. It has been shown that diazepam in a dose of 10 mg/kg i.p. decreases aminooxyacetic acid induced GABA accumulation by about 35% in rat nucleus caudatus during 60 min of exposure to diazepam [18]. In mouse cortex a dose of 10 mg diazepam/kg decreased gabaculine induced GABA accumulation by 67% during 75 min of exposure to diazepam [19], an effect which was clearly dose dependent. The method using labelled precursors showed that GABA turnover was reduced by 65% in rat neostriatum after 1 mg/kg diazepam [9]. It was shown, however, that diazepam depressed the rate of labelling of both glutamate and GABA from glucose [28]. This shows that diazepam in this model affects both the precursor for GABA synthesis as well as the synthesis of GABA. This obviously makes interpretation of the results obtained with the labelled precursor method difficult.

Morphine induced a decrease in GABA turnover measured both with the GABA-transaminase inhibition method (Table 1), and by using labelled precursors [5] (Table 4), by 17% and 65% with the two methods respectively. For morphine, the change in rate constant for GABA efflux (kGABA) was due to the ^{13}C enrichment of GABA only, and not glutamate [5]. Our study with GVG failed to indicate a dose dependency for morphine in the dose range studied. This contrasts with the work on labelled glucose [5], which showed a clear dose dependent GABA turnover in rat neostriatum in the dose range 10–40 mg morphine per kg. Different time of exposure may explain the difference.

We were not able to detect any change in the post mortal GABA accumulation after diazepam or morphine treatment.

Both the GABA-transaminase inhibition method and the *post mortem* method did detect an effect of hypoglycemia on GABA turnover rate. Since we detected a decrease in GABA turnover during hypoglycemia with the *post mortem* method, and not any effect of diazepam and morphine, which have completely different mechanisms of action, we conclude that normal regulation of GABAergic transmission is not present *post mortem*.

Due to the factors discussed above, it is not surprising that the three different methods discussed give both quantitatively different results and have different ability to detect drug-induced GABA turnover changes.

The GVG method can be said to be satisfactory, but has its weakness in the incomplete inactivation of GABA-transaminase, making the measurements comparative only. Further, it requires the effect of the drug to last over at least 1 hr to give reliable data.

The method with labelled precursors gave qualitatively the same results as the GVG method, but has its major weakness in that the kinetic model disregards the compartmentation of amino acids and that the changes in the level of glutamate pool may lead to erroneous conclusions with regard to GABA turnover.

The *post mortem* GABA accumulation seems to be able only to detect effects of drugs associated with the changes in the level of the precursor.

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