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Simultaneous determination of basal and evoked output levels of aspartate, glutamate, taurine and 4-aminobutyric acid during microdialysis and from superfused brain slices

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Abstract

A HPLC method, involving pre-column derivatisation with *o*-phthalaldehyde and fluorescence detection, is described. It allows the resolution of aspartate, glutamate, taurine and GABA, in a single run with detection limits of 3.2, 1.7, 1.4 and 2 fmol/μl of perfusate, respectively. It is sufficiently sensitive and rapid (15 min) for the determination ''on line'' of the four amino acids in perfusates obtained during in vivo microdialysis experiments. The procedure has been used to determine basal, K^+- or veratridine-stimulated release of these amino acids in different brain areas during microdialysis and from perfused tissue slices.

Keywords: Aspartate; Glutamate; Taurine; 4-Aminobutyric acid

1. Introduction

The neostriatum is a complex area of the brain, in which the presence of many different neurotransmitters makes it difficult to study their mutual interactions. The striatonigral neurones, shown to contain the well-established inhibitory neurotransmitter GABA and possibly taurine [1] are controlled by an extensive excitatory corticostriatal projection system which uses the excitatory amino acids, aspartate and/or glutamate. The importance of interactions between these different amino acid systems in the functions of the basal ganglia is now becoming increasingly recognised (see [2–4]), necessitating detailed studies of their interrelationships.

Although HPLC methods for the separation of amino acids, using pre-column *o*-phthalaldehyde (OPA) derivatisation, have been developed (see e.g. [5–10]), none of these methods provides a satisfactory separation of these four amino acids in a single chromatogram with chromatographic run-time short enough to allow the analysis of microdialysis samples ''on line'', as they are collected during the experiment. Furthermore, their sensitivities are, in general, too low to allow the satisfactory determination of GABA outflow in such experiments.

The present work describes a sensitive and rapid HPLC method, using *o*-phthalaldehyde (OPA) derivatisation and fluorescence detection, for the simultaneous measurement of aspartate, glutamate,

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taurine and GABA in perfusates obtained, either in vitro, from superfusion of tissue slices, or in vivo, from microdialysis experiments.

This method has been applied to the measurement of the basal and K^+- or veratridine-stimulated release of these four amino acids from perfused neostriatal and nigral tissue slices and in the extracellular fluid during microdialysis of several brain areas.

2. Experimental

All experiments involving laboratory animals were performed according to the Italian Guidelines for animal care (D.L. 116/92), which were also in accordance with the European Communities Council Directives (86/609/EEC).

2.1. Preparation and superfusion of neostriatal slices

Slices were prepared from male Sprague-Dawley rats (250–350 g) by bilateral dissection of the area of interest from coronal sections (0.3 mm thick), as described previously [1]. The medium used for superfusion was a Krebs Ringer solution (Krebs) containing: 118 m*M* NaCl, 1.2 m*M* KH_2PO_4, 4.7 m*M* KCl, 1.1 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$, and 11.1 m*M* glucose, kept at 37°C under a mixture of 5% carbon dioxide and 95% oxygen. Either one or two slices of neostriatum (STR; 1.9 ± 0.1 mg, $n=64$) or 1–3 slices of substantia nigra pars reticulata (SNr; 0.508 ± 0.013 mg, $n=313$) were added to each superfusion chamber of 0.5-ml volume. In each experiment eight chambers were run in parallel and slices were randomly allocated to each chamber. The slices were superfused through the bottom of the chamber at a flow-rate of 0.3 ml min^{-1}.

2.2. Microdialysis experiments

Male Wistar rats, weighing 250–270 g, were used for vertical and transverse microdialysis experiments. The rats were anaesthetised with chloral hydrate (400 mg/kg, i.p.). The experiments involving vertical microdialysis of STR, globus pallidus (GP) and SNr were performed as described previously [11,12]. Single-cannula microdialysis probes were implanted vertically either into the right STR (3-mm probe tip), GP (2-mm probe tip) or SNr (1-mm probe tip). Stereotaxic coordinates, derived from the atlas of Paxinos and Watson [13], were as follows: STR, AP+0.7 mm, L−3.2 mm and V−5.5 mm; GP, AP−1 mm, L−3.2 mm and V−7.6 mm; SNr, AP−5.4 mm, L−2.2 mm and V−8.9 mm, relative to bregma and dural surface. Transverse microdialysis studies of cortex, hippocampus, septum and STR were performed as described previously by Giovannini and coworkers [14,15] and Blandina et al. [16]. Microdialysis probes were inserted transversely into the cortex, septum, hippocampi and neostriata (8, 2, 6 and 7 mm of exposed membrane, respectively). Stereotaxic coordinates, were as follows: cortex, AP−0.5 mm and H 2.0 mm; septum, AP+0.9 and H−5 mm; hippocampi, AP−3.3 mm and H−3.3 mm; and neostriata, AP 0.0 mm and H−5 mm, from bregma. The experiments were performed with conscious, freely-moving rats, 24 h after implantation of the probes. The probes were perfused with artificial CSF, containing, 140 m*M* NaCl, 3 m*M* KCl, 1.2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.27 m*M* NaH_2PO_4 and 7.2 m*M* glucose (pH 7.4), via polyethylene tubing connected to a microinfusion pump (CMA/100, CMA/Microdialysis AB, Stockholm, Sweden), at a rate of 2.0 μl/min, for both vertical and transverse microdialysis. Following a 1-h stabilisation period, perfusate fractions were collected every 20 min over a 4–5 h period. After three fractions (time −60, −40 and −20 min) had been collected to determine basal levels, the compound under test was included in the superfusing medium for 20 min (one fraction, time 0) and the changes in released amino acid levels were determined in the fractions collected for another 80 min.

2.3. Amino acid analysis

A modification of the HPLC amino acid analysis procedure described by Lenda and Svenneby [6] was used as the starting point for the development of the new procedure.

A gradient liquid chromatograph Shimadzu LC-10AD (Shimadzu Europe, Duisburg, Germany), with a Rheodyne injector (Cotati, CA, USA, Model 7125)

and a 5-μm reversed-phase Nucleosil C_{18} column (250×4 mm; Machery-Nagel, Duren, Germany) kept at room temperature was used. Column effluent was monitored with a Shimadzu spectrofluorimeter RF AXL, equipped with a 18-μl flow-cell, using an excitation wavelength of 340 nm (slit 5) and an emission wavelength of 455 nm (slit 5). A Shimadzu Chromatopac C-R4A integrator was connected to the detector.

The mobile phase consisted of methanol and potassium acetate (0.1 *M*, pH adjusted to 5.52 with glacial acetic acid). All solvents were filtered through 0.22 μm Millipore filters and degassed by sonication for 5 min before use. The volume of sample injected was 20 μl in all cases.

Calibration curves were prepared by adding to blank perfusion fluids, acidified Krebs or CSF for the in vitro or the in vivo conditions, respectively, different amounts of a stock solution of the complete amino acids in 0.05 m*M* HCl, each at 0.5 m*M*, obtained by dilution of the full Pierce standard solution (2.5 m*M*, Pierce, Rockford, IL, USA), and different concentrations of taurine, GABA, and when appropriate, asparagine (ASN) and tryptophan, together with a constant concentration of cysteic acid (CYS AC) as the external standard.

The accuracy and precision of the HPLC method were estimated, by regression analysis according the method of inverse prediction, from 9–15 replicate analyses of known amino acid concentrations added to the appropriate media.

The OPA-reagent for pre-column derivatisation was prepared by addition of 5 mg of OPA and 20 μl of methanol plus 5 μl of 2-mercaptoethanol to 5 ml of 0.5 *M* $NaHCO_3$, pH 9.5, to give a final OPA concentration of 1 mg/ml (7.5 m*M*).

2.4. Statistical analysis

For statistical group comparison, the mean area (per unit fraction) under the concentration–time curve before (basal area) and after stimulation (stimulated area) were calculated. Data were analysed with repeated measures analysis of variance (MANOVA) using the SPSS programme for PC (SPSS Inc., Chicago, IL, USA). The 95% confidence intervals of data shown in Fig. 2 were calculated according to the Fieller's Theorem [17].

3. Results

3.1. Chromatographic conditions

A gradient of two linear steps from 20% to 45% methanol in 100 m*M* potassium acetate pH 5.50, as described by Lenda and Svenneby [6], did not separate taurine from GABA or asparagine from glutamate and the sensitivity was inadequate, especially for GABA (20 pmol injected), for determining basal release from endogenous pools. Optimum resolution and sensitivities were obtained by using (1) a gradient of three linear steps, from 25% to 90%, (2) a lower flow-rate (0.9 ml/min instead of 2 ml/min), (3) a pH of the mobile phase of 5.52, and (4) column at room temperature, (5) modified OPA-derivatisation conditions (see below).

The first and second steps, from 25%–43% methanol (1 min) and from 43%–70% methanol (10 min), resolved the external standard CYS AC (t_R=2.80 min), aspartate (t_R=5.08 min), asparagine (t_R=7.41 min), glutamate (t_R=7.71 min). Serine, histidine, glutamine and OPA-reagent then coeluted in one peak (t_R=8.18 min) and glycine, arginine and threonine coeluted in one peak (t_R=9.62 min) before taurine (t_R=10.28 min), tyrosine (t_R=10.53 min) and alanine (t_R=10.97) followed by the well-separated peaks of GABA (t_R=11.49 min) and tryptophan (t_R=12.04). Methionine, valine and phenylalanine were eluted in the third linear step (1 min), during which methanol was increased from 70% to 90% and during the subsequent isocratic hold at 90% methanol (1 min). Isoleucine, leucine and lysine coeluted during the final step, from 90% back to 25% methanol (1 min). An isocratic hold (1 min) followed, after which time another run could be started immediately, thus the total run time was 15 min. The resolution of aspartate, glutamate, taurine and GABA, each as a single peak, was confirmed both by running standards and samples to which these amino acids had been added.

A typical chromatogram is shown in Fig. 1A. Fig. 1B shows the elution of an OPA-derivatised basal dialysate sample (30 μl), obtained during a microdialysis experiment where a vertical probe was implanted into the globus pallidus. Under these conditions, where the pH of the mobile phase was 5.52, endogenous asparagine (t_R=7.41) was well

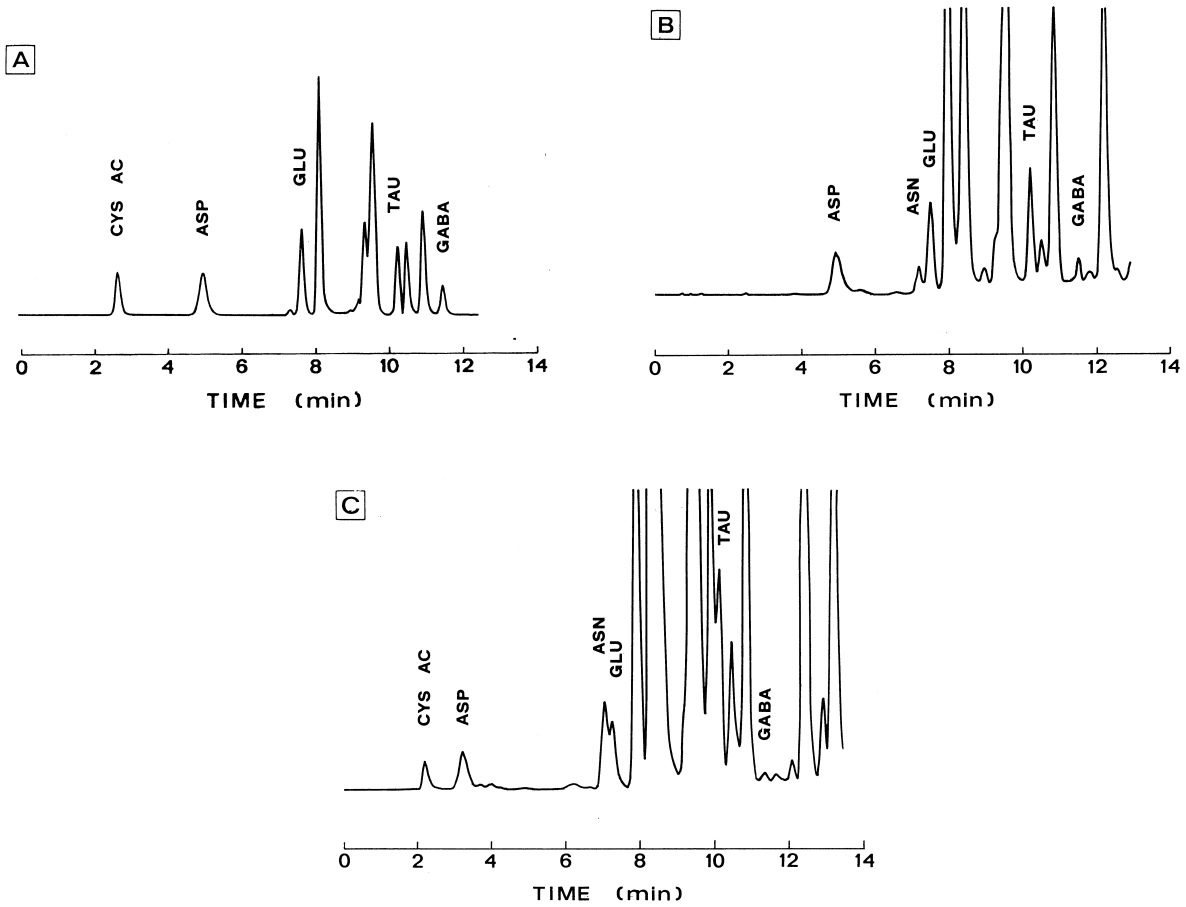

Fig. 1. Resolution and detection of aspartate, glutamate, taurine and GABA by HPLC. Panel A: A typical chromatogram obtained injecting 50 μl of an OPA-derivatised CSF sample containing 0.048 μ*M* GABA, 0.19 μ*M* taurine, 0.59 μ*M* CYS AC (external standard) with the other standard amino acids, each at a concentration of 0.25 μ*M*. The peaks of CYS AC, aspartate (ASP), glutamate (GLU), taurine (TAU) and GABA are labelled. The retention times and composition of the other peaks are given in the text. Panel B: HPLC separation of an OPA-derivatised basal dialysate sample (30 μl) without CYST AC, obtained by vertical microdialysis of the GP. Further to the peaks described above, the well-separated peak of endogenous ASN is indicated. Panel C: HPLC separation of an OPA-derivatised basal dialysate, collected by vertical microdialysis of the SNr, eluted with a mobile phase at pH 5.55 instead of 5.52. At this pH the retention-times were 2.18 and 3.22 for CYS AC and ASP, 7.02 and 7.24 for ASN and GLU, which failed to be separated, 10.45 for taurine, also not separated from other previous peaks, and 11.33 for GABA.

separated from glutamate (t_R=7.71), and endogenous tryptophan (t_R=12.04) was well separated from GABA (t_R=11.49). In contrast, a mobile phase pH of 5.55, as used by Geddes and Wood [18], rather than 5.52, failed to separate glutamate from asparagine and taurine from the previous peaks (Fig. 1C).

3.2. Optimisation of the derivatisation procedure

Since samples obtained from in vitro and in vivo perfusions require different manipulations before derivatisation, the behaviour of simulated in vitro and in vivo perfusates, prepared by the addition of standard amino acids to the appropriate media, was

studied. All derivatisation experiments were performed at room temperature using appropriate amounts of an OPA reagent containing 7.5 m*M* OPA in 0.5 *M* NaHCO3, pH 9.5 and 1 μl ml^{-1} 2-mercaptoethanol.

For studies under in vitro conditions, where slices of fresh tissue were perfused with Krebs at a flow-rate of 0.3 ml/min and fractions were collected every 4 min, it was necessary to store them to await analysis. Samples (1.2 ml) could be stored at −20°C for several months after addition of 5% 0.5 *M* HCl to bring them to pH 4. In order to increase the sensitivity, the samples could be lyophilised or evaporated to dryness under vacuum and taken up into 30 μl of Krebs before derivatisation. Control experiments, in which authentic samples of the four amino acids were added to acidified Krebs, indicated a 70% recovery from the lyophilisation process, after which the dried samples could be stored for over a month without significant losses as a result of instability. When the ratio sample/OPA-reagent (v/v) was decreased from 1/0.33 to 1/4, corresponding to an increase in OPA concentrations from 1.87 m*M* to 6.00 m*M*, and the reaction time was 1.5 min, a statistically significant increase of the fluorescence response was observed only in the case of the aspartate and glutamate derivatives (data not shown). Fig. 2A shows the fluorescence response of the OPA-derivatives as a function of the reaction time, using an OPA concentration of 5 m*M*. In the case of glutamate and, to a lesser extent, aspartate, the fluorescence response increased to a maximum at 5 min, and subsequently declined gradually. The fluorescence of the taurine and GABA derivatives, however, did not significantly change between 0.5 and 1.5 min but subsequently decreased. The procedure adopted for samples obtained under in vitro conditions was, therefore, to add twice the volume of OPA reagent, corresponding to an OPA concentration of 5 m*M*, and to use an incubation time of 1.5 min.

Under in vivo conditions, where microdialysis samples were collected every 20 min whilst perfusing at a flow-rate of 2 μl/min, a volume of only 40 μl CSF was available. Samples of such a small volume could not easily be concentrated. Furthermore, a relatively small volume of the derivatising reagent had to be added in order to avoid sample dilution. When the ratio (v/v) between the sample

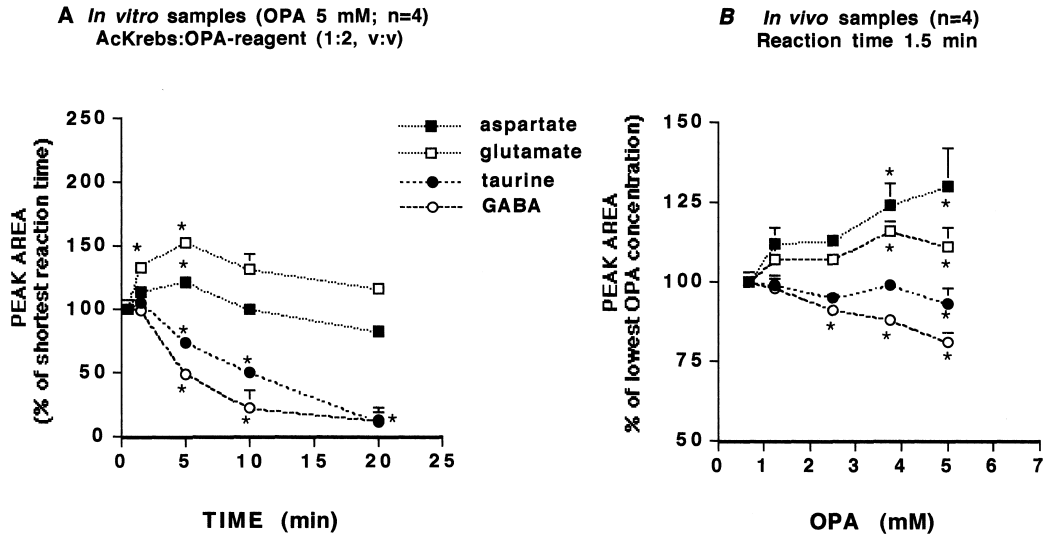

Fig. 2. Effect of OPA-reaction time under in vitro conditions (panel A) and of OPA concentration in in vivo microdialysis experiments (panel B) on the fluorescence response of the OPA-derivatives of aspartate, glutamate, taurine and GABA. Figures are mean values of peak area percentages±s.e.m. * Significantly different ($P<0.05$) from 100% values as indicated by non-overlapping 95% confidence intervals.

and the OPA-reagent was increased from 1/0.1 to 1/2, corresponding to OPA concentrations from 0.68 m*M* to 5 m*M* (Fig. 2B), and the time of reaction was increased from 0.5 to 20 min (data not shown), the fluorescent response of derivatised aspartate and glutamate showed a trend towards an increase with both OPA concentration and time. In contrast, the fluorescent response of taurine and GABA showed a decreasing trend with both parameters. The fluorescence decrease with OPA concentration was statistically significant only in the case of GABA, with a decrease of 20% ($P<0.05$) being observed when the concentration of OPA was increased to 5 m*M*. The decrease in fluorescence observed with time was statistically significant for both taurine and GABA derivatives, from 10 and 5 min onwards, respectively, with the levels at 20 min reduced to 67% and 44%, respectively, of the value observed at 0.5 min.

The procedure adopted for samples obtained under in vivo conditions was to use an OPA concentration of 0.68 m*M*, by adding 4 μl of the OPA-reagent to the 40 μl sample and to inject 20 μl of this on to the HPLC column after an incubation time of 1.5 min.

3.3. Amino acid analysis

The calibration curves exhibited a good linearity for the four amino acids examined, over the concentration range used, showing intersections with the *y*-axis not significantly different from the origin. Means and average standard deviations of slopes for standard curves obtained on different days, using acidified Krebs as blank under the in vitro derivatisation conditions using the external standard, are shown in Table 1. Results obtained with CSF, under the in vivo derivatisation conditions, were not significantly different. Furthermore, the linearity of the calibration curves was not significantly affected by omission of the external standard. As shown in Table 2, the average between-assay coefficients of variation (CV) were below 10% and the difference between found and nominal concentrations ranged from −5.4% to +8.9%, except for the minimum detectable concentrations. The minimum detectable concentrations, 3.2, 1.7, 1.4 and 2 fmol/μl of perfusate for aspartate, glutamate, taurine and GABA, respectively, in spite of a lower degree of accuracy and precision (see Table 2), were significantly different from blank values, as shown by their 95% confidence intervals which did not include zero. The within day CV values were below 2%.

3.4. Basal and K^+-stimulated outflow of endogenous amino acids from different brain regions in vivo

The basal and (100 m*M*) K^+-stimulated outflow of endogenous aspartate, glutamate, taurine and GABA from cortex, hippocampus, septum and STR, during transverse microdialysis experiments, and from STR, GP and SNr, during vertical microdialysis experiments, are shown in Table 3. Basal levels of the four amino acids remained constant over the 4–5 h collection period. Time courses of K^+-evoked (100 m*M*) release of these amino acids from STR, GP and SNr are shown in Fig. 3.

In vertical microdialysis experiments, the mean in vitro membrane recovery for each of the four amino

Table 1
Concentration range, mean of slopes and their average standard deviation (SD) for calibration curves obtained on different days from blank samples[a] to which 4–6 concentrations of aspartate, glutamate, taurine and GABA were added

Amino acid	Concentration range (fmol μl^{-1})	Slope (10^3)			*r*
		N^b	Mean	Average SD	
Aspartate	25–750	18	1.0	0.06	>0.99
Glutamate	25–750	18	1.3	0.07	>0.99
Taurine	20–1.500	29	4.0	0.21	>0.99
GABA	28–1.140	27	3.2	0.15	>0.99

[a] Results obtained with ''spiked'' acidified Krebs including the external standard, under the in vitro derivatisation conditions.
[b] *N*, number of calibration curves.

Table 2
Accuracy and precision of the method using 9–15 replicate analyses of blank samples[a] to which different concentrations of aspartate, glutamate, taurine and GABA were added

Amino acid	Precision concentration (fmol μl^{-1})			Accuracy CV (%)	Minimum detectable concentration (fmol μl^{-1})
	Nominal	Found	Difference (%)		(95% confidence limits, $n=5$)
Aspartate	3.20	3.81	19.1	20.2	3.2 (2.60 – 3.66)
	22.50	24.50	8.9	9.3	
	752.00	740.00	−1.6	2.8	
Glutamate	1.70	1.92	12.9	18.6	1.7 (1.06–2.32)
	22.50	21.75	−3.3	9.9	
	752.00	745.00	0.9	4.6	
Taurine	1.40	1.62	15.7	16.1	1.4 (1.04–1.74)
	22.60	21.38	−5.4	6.7	
	1510.00	1508.00	−0.1	2.9	
GABA	2.00	1.93	−3.5	11.0	2.0 (1.80–2.34)
	28.50	27.70	−2.8	6.8	
	1139.00	1117.00	−1.9	2.2	

[a] Results obtained with ''spiked'' blank CSF, without the external standard, using the in vivo derivatisation conditions.

acids was 25–30±4%, 14–17±2% and 6–8±1% for STR, GP and SNr, respectively, in agreement with the results of other authors using a similar length of exposed membrane [19–25]. In transverse microdialysis experiments the mean recovery was 38–48±8% [14,26]. The recovery experiments were performed at room temperature. Values reported in tables and figures were not corrected for recovery.

The effect of tetrodotoxin (TTX) on the release of the four amino acids (Fig. 4) and of nipecotic acid on the release of GABA (data not shown) were studied in the STR and SNr in vivo. The application of the GABA–uptake inhibitor, nipecotic acid (100 μM, through the microdialysis probe) produced a statistically significant increase ($P<0.05$) in the extracellular level of GABA, in the STR (415%) and SNr (563%) but did not significantly modify the outflow of the other amino acids. TTX (3 μM) induced a statistically significant decrease to 39% of basal output of aspartate in the STR but not in the SNr. The decrease of glutamate to 80% of basal output induced by TTX reached statistical significance in the STR but not in the SNr. Basal GABA output was significantly reduced to 50% in STR and to 60% in SNr. Taurine levels also showed a statistically significant decrease down to 77% in both the STR and the SNr.

3.5. Basal and evoked outflow of endogenous amino acids from perfused neostriatum and substantia nigra slices

The presence of veratridine in the perfusion fluid for a period of 4 min (one fraction) induced a concentration-dependent increase in the release of the four amino acids in neostriatal slices. Release values under basal conditions (mean of three pre-stimulation fractions) and following stimulation (values at peak) by different concentrations of veratridine are shown in Table 4. TTX (3 μM) abolished the release of these amino acids induced by 50 μM veratridine. A statistically significant increase of the release of these amino acids in substantia nigra slices was also evoked by 50 mM K^+ (Table 4).

4. Discussion

Although the procedure reported here was developed from that of Lenda and Svenneby [6], that method did not give adequate separation of aspartate, glutamate taurine and GABA. Furthermore, its detection limits were inadequate for monitoring basal extracellular levels, especially under in vivo microdialysis conditions, where the recovery of the

Table 3
Extracellular levels of aspartate, glutamate, taurine and GABA monitored in different brain areas by in vivo transverse and vertical microdialysis, under basal and K^+-depolarising (100 m*M* KCl) conditions in freely moving male rats[a]

Brain area–amino acid	Basal release (n*M*)	KCl-evoked release (n*M*) (Peak as % of basal)
Transverse microdialysis		
Cortex (n=8)		
aspartate	467±49	1353±248 (290)
glutamate	1328±268	3864±710 (291)
taurine	4000±158	9739±843 (243)
GABA	307±58	2729±333 (889)
Hippocampus (n=8)		
aspartate	952±46	2220±152 (233)
glutamate	7016±785	13 307±1572 (190)
taurine	4552±301	17 654±1422 (388)
GABA	114±22	1250±179 (1096)
Septum (n=6)		
aspartate	980±90	2250±140 (230)
glutamate	5680±480	11 660±910 (205)
taurine	3720±560	9370±1280 (252)
GABA	244±53	1762±427 (722)
Neostriatum (n=7)		
aspartate	1413±300	2130±421 (151)
glutamate	8712±1,150	13 860±1830 (159)
taurine	6,573±1,031	12 241±1822 (186)
GABA	141±30	2869±307 (2,035)
Vertical microdialysis		
Neostriatum (n=15–18)		
aspartate	215±22	522±77 (243)
glutamate	472±39	1589±159 (337)
taurine	1018±96	2952±453 (290)
GABA	29±4	346±64 (1193)
Globus Pallidus (n=13–17)		
aspartate	195±24	624±81 (320)
glutamate	327±66	855±162 (261)
taurine	651±171	2439±317 (375)
GABA	25±4	639±121 (2556)
Substantia Nigra reticulata (n=15)		
aspartate	255±34	754±138 (296)
glutamate	553±76	1623±263 (293)
taurine	866±136	2385±276 (275)
GABA	31±3	1524±406 (4916)

[a] Statistical analysis performed on values of the area under the concentration–time curve, basal area (from −60 to 0 min) and stimulated area (from 0 to 100 min). In all areas K^+ induced a statistically significant increase in the output of the four amino acids (stimulated area higher than basal area, $P<0.05$, MANOVA). *n*, number of animals.

amino acids through the membrane of the vertical probe is very low (e.g. 6–9% for SNr). High sensitivity is essential for detecting the low basal striatal and nigral GABA levels (0.3–0.5 pmol/20 μl of perfusate). The method described here has a good sensitivity for GABA, 40 fmol/20 μl of dialysate sample (2 n*M*). It is also sufficiently rapid for the determination of these four amino acids in perfusates

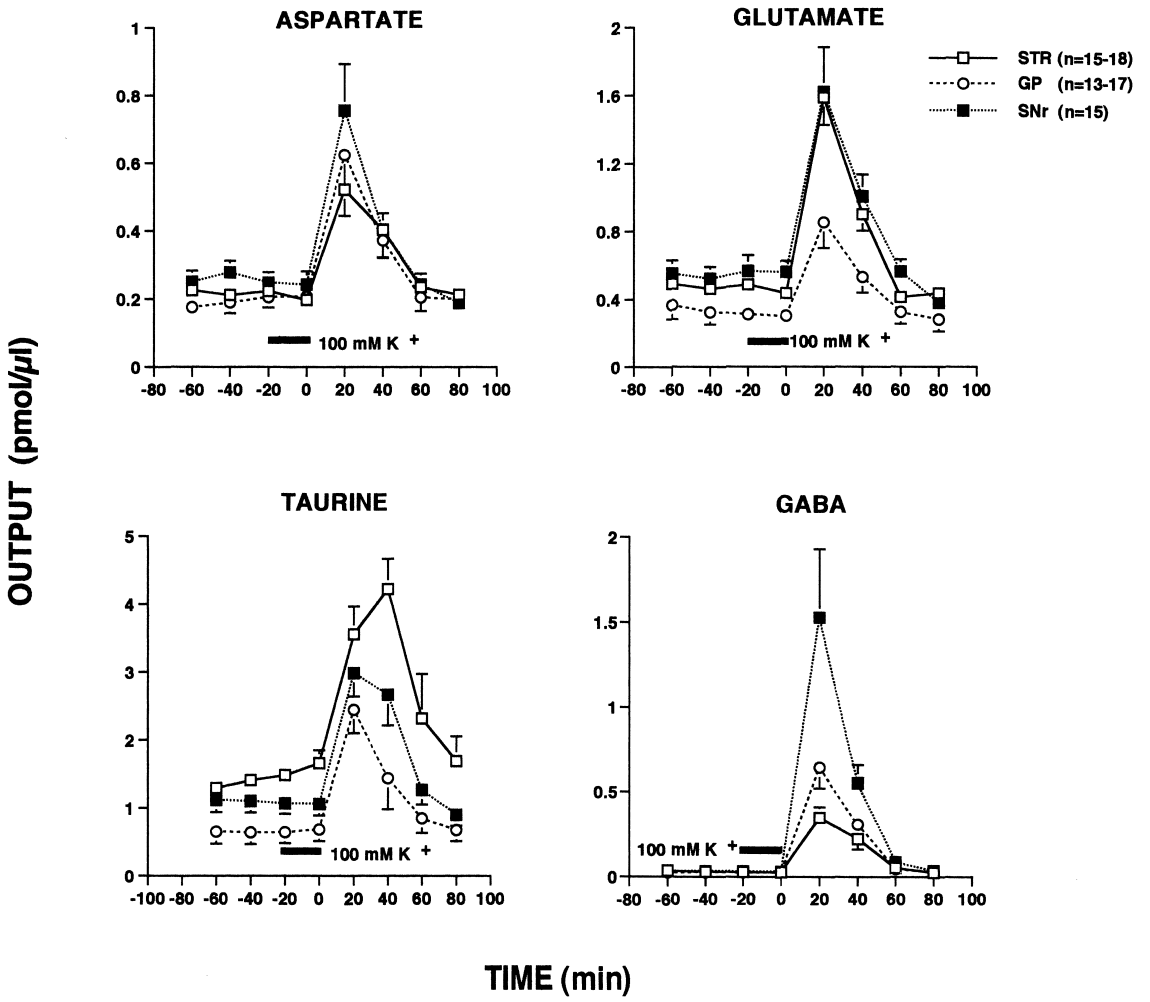

Fig. 3. Time-course of the basal and K^+-evoked release of aspartate, glutamate, taurine and GABA from neostriatum (STR), globus pallidus (GP) and substantia nigra pars reticulata (SNr). Figures represent mean±s.e.m. (pmol/µl, *n*=number of animals). Solid bar indicates time of K^+ application.

obtained in vivo at the time of collection. The minimal sample manipulation necessary also reduces possibilities of experimental error.

None of the previously-reported HPLC procedures, which have used fluorescence or electrochemical detection, effect the resolution of each of these four amino acids in a single HPLC run with sufficient sensitivity (see e.g. [5,10,27–31]). Even in the most recent published work, the analysis of GABA was performed separately from that of aspartate/glutamate (see e.g. [24,32,33]). Indeed, the sensitivity of the present procedure is comparable to or lower than that of most methods exclusively dedicated to GABA detection [34–36]. OPA-isoindole derivatives of the amino acids are rather unstable, with the time-courses of their decay depending on the nature of the amino acid as well as upon the amount of OPA and the pH of the medium used to derivatise them (see e.g. [5]). Substantial changes to the original method were necessary to obtain adequate separation and sensitivity. The critical dependence of the separation on pH is illustrated in Fig. 1C by the observation that resolution is diminished by an increase of pH from 5.52 to 5.55. As reported by Jarrett et al. [8]

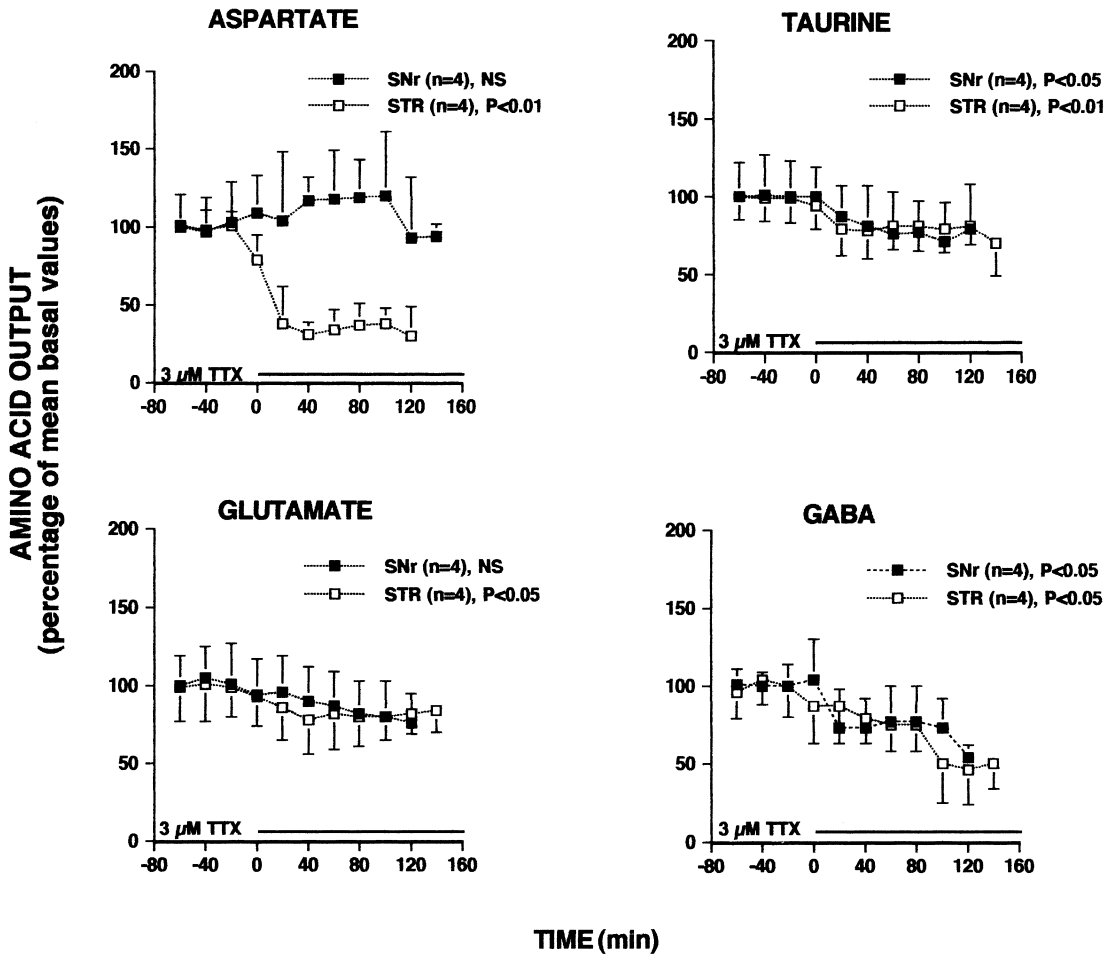

Fig. 4. Effect of TTX (3μ*M*, 0–120 min) on the basal release of aspartate, glutamate, taurine and GABA from neostriatum (STR) and substantia nigra (SNr) in vivo in freely-moving rats. Figures represent amino acid output expressed as percentage of mean basal (pre-TTX fractions). Vertical bars represent s.e.m.. Original output values (pmol/μl) were used for statistical analysis. *P* values indicate probability level of significance of the difference between the mean stimulated area (in presence of TTX, from 0 to 120 min) and the mean basal area (pre-TTX, from −60 to 0 min) using the paired *t*-test. *n*=number of animals.

all amino acids, but especially those containing a carboxylic acid side chain, are eluted at shorter times as the pH is increased. This may be due to a decrease in the protonation of the acid side chain resulting in decreased interactions with the C_8 stationary phase and decreasing retention times. However, since the fluorescence intensity also decreases as pH decreases [8], the optimum conditions represent a balance between resolution with short enough retention times to give sharp elution peaks and sensitivity. Indeed values of pH below 5.52 had the disadvantage of longer retention times with lower detection sensitivity. For example at pH 5.3 the t_R of the glutamate-derivative was already 130% of that at pH 5.52 and was not resolved from the subsequent OPA-peak.

Studies on the endogenous release of these amino acids from substantia nigra slices have been restricted by the lack of sensitivity of the available methods. Very few data are available for GABA [37,38] or for aspartate and glutamate [39].

As shown in Table 2, the sensitivity of the method described here is sufficient to allow the release of these amino acids from endogenous stores to be studied without the necessity of pre-loading. Pre-

Table 4
Extracellular levels of aspartate, glutamate, taurine and GABA monitored in vitro by perfusion of rat neostriatal and substantia nigra slices, under basal conditions and following veratridine (VER) stimulation and TTX

Brain area Treatment–amino acid	Basal release (fmol/min/mg w.t.)	Evoked release at peak (fmol/min/mg w.t.) (% of basal)
Neostriatum		
Control (n=12)		
aspartate	215±42	213±29 (99)
glutamate	306±51	328±56 (107)
taurine	663±134	751±126 (113)
GABA	109±11	116±22 (106)
VER 7.5 μ*M* (n=4)		
aspartate	237±20	276±14 (116)
glutamate	235±30	463±59[a] (197)
taurine	640±126	824±175 (129)
GABA	98±26	168±27 (171)
VER 25 μ*M* (n=4)		
aspartate	239±54	874±208[a] (366)
glutamate	256±19	5759±1663[a] (2,250)
taurine	752±165	1981±596[a] (263)
GABA	87±4	1308±371[a] (1,503)
VER 50 μ*M* (n=13)		
aspartate	213±48	2312±463[a] (1,085)
glutamate	384±90	9564±1,888[a] (2,491)
taurine	782±181	2589±362[a] (331)
GABA	112±26	2586±516[a] (2,309)
VER+TTX 3 μ*M* (n=8)		
aspartate	358±82	440±81 (123)
glutamate	327±121	453±157 (138)
taurine	800±124	898±97 (112)
GABA	93±26	103±28 (111)
Substantia Nigra		
K^+ 50 m*M* (n=4)		
aspartate	174±17	478±38[a] (275)
glutamate	155±25	2,236±208[a] (1,442)
taurine	417±17	1,670±120[a] (400)
GABA	22±3	376±59[a] (1,709)

[a] Stimulated area significantly higher ($P<0.05$, MANOVA) than basal area. Values are expressed as means±s.e.m., n, number of animals. Statistical analysis performed on values of the area under the concentration–time curve, basal area (from −8 to 0 min) and stimulated area (from 0 to 20 min).

loading has been extensively used to increase the sensitivity in studies with tissue preparations and it has also been used prior to microdialysis studies [40]. However, it may induce artefacts due to adventitious uptake into, and subsequent release from, extraneous sites (see e.g. [41,42]). Furthermore, metabolic transformations can complicate the results of pre-loading experiments. For example, glutamate pre-loading will result in the formation of GABA in amounts that will depend on the conditions used, whereas in studies involving GABA pre-loading it has proven necessary also to include an inhibitor of GABA transaminase to prevent its metabolism [43]. In the latter case aminooxyacetic acid has normally been used, although this compound does not have a high specificity as an enzyme inhibitor and can affect the activity of some other transaminases as well as that of glutamate decarbox-

ylase [44]. Aminooxyacetic acid has also been shown to affect the release of GABA itself [45–47]. For these reasons it has not proven possible to study the behaviour of slice systems after simultaneous pre-loading of all four of these amino acids. Several authors have also used an uptake blocker of the neurotransmitter to increase the sensitivity of the pre-loading method. However, this can influence the response of the neurotransmitter system to pharmacological or physiological manipulation and may, therefore, give inaccurate information about the control of endogenous release [48].

The basal in vivo levels of GABA, taurine, glutamate and aspartate determined by vertical microdialysis in the present work (Table 3) are in agreement with those reported in the literature. In the case of glutamate and aspartate it is worth noting that, while the present ratios of glutamate to aspartate are similar to those reported by most other workers (1.7–3.4) [19,21–22,27,49–51], ratios which were much higher (9 and 18, in the STR and SNr of the halothane-anaesthetised rat, respectively, and 41 in the STR of the awake rat) were reported by Herrera-Marschitz et al. [24], who used a single chromatographic procedure to determine these two amino acids. A similarly high ratio of 29 was reported in STR by You et al. [52], who used a procedure that was essentially the same as that used by Herrera-Marschitz et al. [24]. The reasons for this discrepancy are unclear, but incomplete resolution of glutamate and asparagine may be a factor. As discussed above, close control of the pH is necessary for adequate resolution and optimum sensitivity. It is also possible that, under non-optimal chromatographic conditions, deamidation of glutamine and asparagine may occur, leading to erroneous results.

The presence of 3 μM TTX in the perfusion fluid induced a statistically significant decrease in the basal release of taurine and GABA in vivo from neostriatum and substance nigra. In contrast, aspartate and glutamate release was significantly reduced by TTX in the STR but was not affected in the SNr. Since a TTX-induced decline in basal levels is generally assumed to be an indication of neuronal origin (see [53]) these results suggest that 61, 20, 23 and 50% of the basal neostriatal release of aspartate, glutamate, taurine and GABA, respectively, and 23 and 40% of the basal nigral release of taurine and GABA, respectively, may have originated from neuronal elements. The lack of any significant response of basal aspartate and glutamate outflow to TTX in the SNr suggests that these amino acids derive from glial cells rather than from neurones under these conditions.

The conclusion that a proportion of the taurine in STR and SNr may be neuronal is consistent with an earlier report of its presence in a subpopulation of striatonigral neurones [1]. The functions of this amino acid, which is present in high concentrations in the brain, are still far from clear. It has been variously suggested to be an osmoregulator, an antioxidant, a membrane-stabiliser, a modulator of trophic factors and/or calcium ions and a neurotransmitter or neuromodulator (see e.g. [54]). The observation in the present work, that the decline in GABA levels resulting from TTX administration was paralleled by a smaller, but significant, fall in taurine levels, might suggest the operation of some compensatory system**,** and further work will be necessary to elucidate the mechanisms underlying this effect. In this context the ability to study the outflow of these amino acids simultaneously, and in identical conditions, should prove to be of particular value.

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