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GLUTAMATE, GABA, GLYCINE AND TAURINE MODULATE SEROTONIN SYNTHESIS AND RELEASE IN ROSTRAL AND CAUDAL RHOMBENCEPHALIC RAPHE CELLS IN PRIMARY CULTURES

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Abstract—Control of serotonin release and synthesis by amino acid neurotransmitters was investigated in rat rostral and caudal rhombencephalic raphe cells in primary cultures respectively. Endogenous amounts of taurine, glycine, GABA and glutamate were measured in both types of cultures. These amino acids were spontaneously released to the incubating medium. Exogenous taurine (10^{-4} M) inhibited release and synthesis of newly formed [^{3}H]serotonin [^{3}H]5-HT from [^{3}H]-tryptophan only in rostral raphe cells. Glycine (10^{-3} M) decreased [^{3}H]5-HT release in both types of cells, synthesis being diminished only in rostral raphe cells. Glycine inhibitory effect was totally blocked by strychnine (5×10^{-5} M). GABA (10^{-4} M) reduced [^{3}H]5-HT metabolism in rostral as well as caudal raphe cells. This effect was totally antagonized in caudal and partially in rostral raphe cells by bicuculline (5×10^{-5} M) a $GABA_A$ receptor antagonist. Baclofen (5×10^{-5} M), a $GABA_B$ receptor agonist, induced a decrease of 5-HT release in rostral raphe cells. These observations suggest that monoamine release was entirely mediated by $GABA_A$ receptors in caudal raphe cells although $GABA_A$ and $GABA_B$ receptors were involved in control of 5-HT metabolism in rostral raphe cells. L-glutamate (10^{-4} M) stimulated 5-HT metabolism in both types of cells, effect totally blocked by PK26124 (10^{-6} M). *N*-methyl-D-aspartate (10^{-4} M) enhanced 5-HT metabolism and the induced-effect was antagonized by the selective *N*-methyl-D-aspartate receptor antagonist D,L-2 amino-5-phosphonovaleric acid. Quisqualate (10^{-5} M) stimulated [^{3}H]5-HT release only in caudal raphe cells. This effect was mimicked by (RS)-a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, a quisqualate "ionotropic" receptor agonist, this increase being blocked by 6,7-dinitroquinoxaline 2,3-dione. These observations suggest that the glutamate stimulating-induced effect on serotonin metabolism is entirely mediated by *N*-methyl-D-aspartate receptor-type in rostral raphe cells and that quisqualate "ionotropic" receptors are also involved in caudal raphe cells. Taken together these results show that [^{3}H]5-HT metabolism is controlled by taurine, glycine, GABA and glutamate in rhombencephalic raphe cells in primary cultures. However, some difference in amino acid receptor-types involved in the control of serotonin metabolism are observed according to the rostral or caudal origin of raphe cells.

The idea has recently gained acceptance that the role of neurotransmitters in interneuronal communications is not restricted to the mature central nervous system (CNS) but is also important during ontogenesis. Thus, other functions have been proposed for these molecules, including a trophic role during brain development. Among the neurotransmitters supposedly modulating cell development are monoamines and more particularly, serotonin (5-HT) which was shown to play an important role in this way (Lauder *et al.*, 1982; Chubakov *et al.*, 1986; Whitaker-Azmitia, 1991).

The concept that amino acids might function as neurotransmitters in the brain arose largely during the 1960s. They are schematically divided into two classes according to their functions: (1) excitatory amino acids (EAA) such as glutamate (GLU) and structurally related compounds (Watkins and Evans,

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Abbreviations: AMPA, (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APV, DL-2-amino-5-phosphonopentanoic acid; CSF, artificial cerebro spinal fluid; DIV, day *in vitro*; DNQX, 6,7-dinitroquinoxaline-2,3-dione; E.A.A., excitatory amino acid; GABA, γ-aminobutyric acid; GLU, glutamic acid; 5-HT, 5-hydroxytryptamine = serotonin; NMDA, *N*-methyl-D-aspartic acid; PK26124, 2-amino-6-trifluoromethoxybenzothiazole.

1981; Fonnum, 1984) on the one hand and (2) inhibitory amino acids such as γ-amino-butyric acid (GABA), glycine and taurine on the other hand (Oja *et al.* 1977; Huxtable, 1989).

The pharmacology and the physiology of these amino acids have been extensively studied in the mature CNS. But in addition to their role in neurotransmission, some amino acids play a function in brain development (McDonald and Johnston, 1990, for review). If EAA are mainly associated with the neuronal death (excessive activation of EAA receptors produces neuronal injury and death) (Regan and Choi, 1991), EAA have also a neurotrophic function during development. Several studies suggest that EAA are involved in various processes during CNS development, including the regulation of neuronal survival and synaptic stabilization (Pearce *et al.*, 1987; Balazs *et al.*, 1988).

Because these neurotransmitter families (amino acids and 5-HT) are involved in brain development, it appeared interesting to determine whether 5-HT metabolism was controlled by these amino acids during early period of development.

Primary cultures of CNS neurons have been extensively used as a convenient model in the field of developmental neurobiology. They provide a model whose environment can be totally controlled and which allows pharmacological manipulations of living cells. In the mature rat brain the different raphe nuclei are far from being an homogeneous population. The 5-HT immunocytochemistry distinguishes two well defined cellular groups in rat embryos at 15 days of gestation: (1) a rostral group, containing B4 B9 nuclei; (2) a caudal group containing B1–B3 nuclei (König *et al.*, 1988). In a previous study, we analyzed some of the mechanisms regulating the 5-HT expression in B4–B9 and B1 B3 in primary cell cultures and we reported that the 5-HT expression in these two groups was differently regulated by their respective lateral alar plates (Becquet *et al.*, 1991). These results motivated the comparative analysis of the control of 5-HT metabolism by amino acids in rostral (B4–B9) vs caudal (B1–B3) rhombencephalon in primary culture.

In the present study we first determined whether amino acids present in the brainstem in mature CNS were also present in our cultures by measuring GLU, GABA, glycine and taurine amounts in cells and in the incubation medium using HPLC coupled to fluorescence detection (Francois-Bellan *et al.*, 1988). Then, to determine how GLU, GABA, glycine and taurine might interact with serotoninergic neurons the effects of natural occuring amino acids have been first tested on synthesis and release of [^{3}H]5-HT newly formed from [^{3}H]tryptophan in 12 day *in vitro* (DIV) cell cultures. The specificity of these effects has been verified by using specific receptor antagonists. Finally, attempts were made to determine the EAA receptor type [*N*-methyl-D-aspartate (NMDA) vs non-NMDA receptors] as well as the GABA receptor type ($GABA_A$ vs $GABA_B$) involved in the control of 5-HT metabolism.

EXPERIMENTAL PROCEDURES

Dissection

Embryos from timed pregnant Sprague Dawley rats (CERJ, Route des Chênes Secs, St Genest, France) at 15 days of gestation (E15, day following insemination = day 0) were aseptically removed from the mother anaesthetized with Nesdonal®. Gestational staging was confirmed using measurement of crown-rump length. The dissection procedure has been described in detail elsewhere (König *et al.*, 1987). Briefly, the part of the head containing the mesencephalon and the rhombencephalon was dissected out in Hank's balanced salt solution. Immunocytochemical studies using antibodies directed against 5-HT reveal that, at E15, all serotoninergic raphe cells are located in rhombencephalon (König *et al.*, 1988). The rhombencephalon was then separated from the mesencephalon. Finally, a section was made at the level of the pontine flexure to separate the rhombencephalon into two parts. The presumed analage of groups B4–B9 of the raphe nuclei are localized in the rostral part of the rhombencephalon, whereas the B1–B3 are localized in the caudal part. (König *et al.*, 1988).

Cultures

The rostral and the caudal groups of raphe nuclei, were mechanically dissociated in F12/DMEM (50/50) medium supplemented with 10% fetal calf serum. Tissue culture dishes were previously coated with D-polylysine (10 μg/ml) for 2 h with medium (DMEM/F12) supplemented with 20% fetal calf serum. Cells were resuspended in serum free medium, (Bottenstein and Sato, 1979) supplemented with 17 β = estradiol (10^{-12} M), progesterone (2×10^{-8} M), triiodothyronine (10^{-9} M), ascorbate (10^{-4} M) and plated at a density of 1.5×10^6 cells/35 mm well (Faivre-Bauman *et al.*, 1984). Six well trays were used. Cultures were maintained at 37 C in an humidified atmosphere of 7% CO_2 in air. Culture medium was changed on day 4 and then every 2 days. After 12 DIV, cells were used either for determination of endogenous amino acid amounts or pharmacological studies on the release and synthesis of 5-HT.

Determination of endogenous amino acid amounts

Incubation procedure. After 12 DIV, the medium was withdrawn and cells were washed twice with 1 ml of artificial cerebro spinal fluid (CSF) in mM: NaCl, 126.5; $NaHCO_3$, 27.5; KCl, 2.4; K_2HPO_4, 0.5; $CaCl_2$, 1.1; $MgCl_2$, 0.85, glucose, 5.9; adjusted to pH 7.3 with an O_2-CO_2, 95:5 v v mixture) and kept at 37 C in an incubator. Sixty min later CSF was withdrawn and poured in tubes. Cell content was extracted with hydrochloric acid solution (0.2 N, 1 ml)

Cells were sonicated, kept overnight at −20°C and then the homogenate was centrifuged (12,000 *g*; 20 min).

Biochemical analysis. Amino acid quantities were determined in CSF and cell extracts respectively using HPLC (pumps Waters 510; Ultrasphere ODS 5 μm, Beckman) and pre-column fluorescence derivatization using *O*-phtaldialdehyde (Francois-Bellan *et al.*, 1988). Elution of amino acid derivatives was achieved using a gradient of sodium acetate buffer (0.1 M; pH 5.5) methanol. The gradient was monitored by a Waters "Automated Gradient Controller" (flow rate: 1 ml/min). The initial phase was composed of 75% natrium acetate buffer and 25% methanol. Fluorescence was detected using a Waters spectrofluorimeter (M420-E). Identification and quantification of glutamic acid, GABA, glycine and taurine were obtained using external standard solutions. Amino acids amounts were expressed as nmol/mg of protein.

Analysis of [^{3}H]5-HT synthesis and release endogeneously formed from [^{3}H]tryptophan

Incubation procedure After 12 DIV cells were washed twice with 1 ml of CSF containing pargyline (5×10^{-5} M). Cells were preincubated for 15 min in 1 ml of CSF and kept at 37 C in the incubator. Freshly purified [^{3}H]TRP (10 μCi; 25–30 Ci/mmol; Amersham Centre, U.K.) was then added to the incubating medium. [^{3}H]5-HT release and synthesis were estimated during a 60 min incubation period of time: in a control condition or in presence of antagonist (60 min) and/or agonist (the last 30 min). After this incubation period, the CSF was removed and analyzed separately. Cold ethanol–water solution (1.5 ml; 74:16 v/v) was rapidly added into each well, [^{3}H]5-HT amounts in cells and in CSF were estimated separately. Plates were then maintained at −20°C for 30 min and cells were disrupted with a teflon scraper and the solution was sonicated. Afterwards homogenates were kept overnight at −20 C. Incubation media were immediately treated for biochemical analysis.

Biochemical analysis. [^{3}H]Serotonin in both the medium and the cells was separated from [^{3}H]TRP and [^{3}H]metabolites by successive ion exchange chromatography.

Cells contents Cell homogenates were centrifuged (12,000 *g*, 20 min, 4 C). The supernatant was diluted with distilled water (v/v) and the pH adjusted to 6.5. The supernatant was passed through an Amberlite CG50 column (0.4 × 2.5 cm high). The resin was washed with KH_2PO_4 (0.05 M, 10 ml) and distilled water containing 0.1% Triton X-100, (5 ml). The [^{3}H]5-HT was then eluted with HCl (3 N, 3 ml).

Incubation medium. The CSF sample (pH 7.3) was chromatographied through a small microcolumn of Dowex AG50 introduced into a plastic tip of an automatic pipette. The resin was washed with 2 ml of solution of 0.2 M sodium acetate and 0.1% Triton X-100 in water. The [^{3}H]5-HT was then eluted with 2 ml of a 2 N perchloric acid–ethanol solution (1.2 v/v). To reduce contamination by the remaining [^{3}H]TRP the Dowex eluate was neutralized (pH 6.7) and passed through an Amberlite CG50 column (0.4 × 1.5 cm). The column was washed with 10 ml ethanol–water (2:1, v/v) and [^{3}H]5-HT was finally eluted with 2 ml of sodium borate buffer (pH 10) (Hery *et al.*, 1979).

The amounts of [^{3}H]5-HT were determined by liquid scintillation counting using 10 ml of Emulsifier Safe™ (Packard)/sample. Blank values were determined by incubating cells at 0 C. The values obtained were corrected for respective recoveries.

Expression of data and statistical analysis

The [^{3}H]5-HT amounts were expressed as nanocurie/milligram of protein (nCi/mg prot). Protein determinations were performed according to Lowry *et al.* (1951) with bovine serum albumine as standard. Results were expressed as % of control values obtained on cells incubated without adding tested compounds. Experiments were performed at least 2 times. Results are the mean ± SEM obtained in 12 samples.

Due to the distribution of our data, statistical analyses were made on absolute values using the Mann–Whitney test. When the *P*-value was higher than 0.05 the difference was not considered to be significant.

Pharmacological agents

Pargyline, GABA, glutamic acid, quisqualic acid, NMDA, aspartic acid, bicuculline, glycine, taurine and strychnine were purchased from Sigma Chemical Co. (St Louis, U.S.A.); AMPA, APV, DNQX from Tocris Neuramin Ltd (Buckhurst Hill, U.K.). RS-baclofen was a generous gift from (Ciba–Geigy, Basel, Switzerland) and PK 26 124 from Rhône-Poulenc Rorer (Vitry sur Seine, France).

RESULTS

Taurine, glycine, GABA and glutamate levels in rostral and caudal raphe primary cell cultures

Endogenous amounts of amino acids in cells and released during 60 min incubation time in CSF were determined in primary cell cultures (12 DIV) from rostral (B4–B9) and caudal (B1–B3) rhombencephalic raphe nuclei.

Taurine. Both rostral and caudal rhombencephalon in cultures contained taurine. The taurine cell content was significantly lower in caudal than in rostral cultures (−27%; $P < 0.05$). Taurine was also detectable in the medium. After a 1 h long superfusion, no significant differences were detected in the two culture conditions. The quantities of taurine in medium represented 7 and 10% of the cell content and a concentration of 3.09 and 1.55 10^{-6} M in rostral and caudal raphe cell cultures respectively (Table 1).

Glycine. Similar levels of glycine were detected in cells in both rostral and caudal rhombencephalon cultures. As compared to the cell content, large amounts of glycine were released in CSF during the 60 min of the incubation period 123 and 105% corresponding to a concentration of 4.66 and 2.45 10^{-6} M in rostral and caudal raphe nuclei respectively. No significant differences were observed in the amount of glycine in rostral vs caudal rhombencephalon, neither in cells nor in medium (Table 1).

GABA. The GABA endogenous contents in cells were the lowest and no statistical differences in rostral vs caudal rhombencephalon were observed. However, the GABA amounts in medium were greater (+34% $P < 0.05$) in rostral than in caudal rhombencephalon, leading to a greater ratio of medium/cell amount in caudal (74%) than in rostral (50%) raphe cell cultures.

Table 1 Amino acids content in cells and medium from rostral and caudal raphe primary cell cultures

	Cells (nmol mg prot)	Medium (nmol mg prot)	Medium (M × 10 ^{6})
Rostral Raphe Nuclei			
GABA	9.27 ± 0.40	4.70 ± 0.25	1.14 ± 0.06
Glycine	15.94 ± 0.52	19.26 ± 0.86	4.66 ± 0.12
Taurine	180 ± 7.09	12.77 ± 0.48	3.09 ± 0.12
Glutamate	28.50 ± 1.59	7.74 ± 0.60	1.87 ± 0.14
Caudal Raphe Nuclei			
GABA	8.77 ± 0.52	6.33 ± 0.45	0.81 ± 0.06
Glycine	18.13 ± 0.94	19.21 ± 0.78	2.45 ± 0.10
Taurine	133.16 ± 9.11	12.08 ± 1.03	1.55 ± 0.13
Glutamate	21.49 ± 1.71	9.85 ± 0.91	1.26 ± 0.12

Cells (12 day in vitro) were incubated in CSF for 60 min. Amounts of GABA, glycine, taurine and glutamic acid in cells and medium were determined using HPLC. Results are means ± SEM of data expressed as nmol mg protein. Values were obtained from 12 samples.

After the 60 min of incubation the concentrations were 1.14 and 0.81 10 ^{6} M in rostral and caudal rhombencephalic cells respectively (Table 1).

Glutamic acid. The GLU cell contents were slightly but significantly lower (−24%; $P < 0.05$) in caudal than in rostral raphe cells. Contrariwise GLU amounts in medium were significantly greater (+27%, $P < 0.05$) in caudal than in rostral rhombencephalon cultures. After 1 h the concentrations were similar in rostral and caudal rhombencephalic cells and corresponded to 1.87 and 1.26 10 ^{6} M respectively (Table 1).

Spontaneous release and synthesis of [^{3}H]5-HT endogeneously formed form [^{3}H]tryptophan in the rostral and caudal rhombencephalon primary cultures

After 12 days of culture, cells were incubated in 1 ml of CSF containing pargyline (5 × 10 ^{5} M) in presence of freshly purified [^{3}H]TRP (10 μCi/ml). After a 60 min incubation the release of [^{3}H]5-HT was determined by measuring the amounts of [^{3}H]5-HT in the incubating medium and synthesis was estimated by the sum of the quantities of [^{3}H]5-HT measured in the cells and in the medium.

The means of the absolute amounts of [^{3}H]5-HT synthetized in control conditions in rostral and caudal rhombencephalon cultures were 510.42 ± 54.36 and 163.33 ± 18.21 nCi/mg protein respectively. The means of the absolute quantities of [^{3}H]5-HT released during the 60 min incubation time from the rostral and the caudal rhombencephalon cultures were 63.12 ± 7.87 and 41.27 ± 5.48 nCi/mg protein respectively.

Effects of taurine on 5-HT metabolism

Application of taurine (10 ^{4} M) in rostral raphe nuclei cultures induced a decrease in 5-HT metabolism. A simultaneous reduction of [^{3}H]5-HT synthesis and [^{3}H]5-HT release (−25%, $P < 0.05$ and −24% $P < 0.05$ respectively) was observed (Fig. 1). Smallest, but not significant, decreases of [^{3}H]5-HT synthesis and release were detected at 10 ^{5} M (−15 and −11% respectively). No effects were observed at 10 ^{6} M.

No significant changes in 5-HT metabolism were induced by taurine at various concentrations used in caudal raphe nuclei cultures (Fig. 1).

Effects of glycine on 5-HT metabolism

As shown in Fig. 2 application of glycine (10 ^{3} M) in rostral raphe nuclei cultures induced a decrease of 5-HT metabolism. Both [^{3}H]5-HT synthesis and [^{3}H]5-HT release were significantly reduced (−25%, and −22%, respectively). In caudal raphe cultures glycine (10 ^{3} M) induced an identical decrease (−22%) in [^{3}H]5-HT release and synthesis (Fig. 2). No inhibitory induced-effects were detected in rostral as well as caudal raphe cells when glycine was used at lower concentration than 10 ^{3} M.

In order to determine whether the strychnine-sensitive glycine receptors were involved in the inhibitory action of glycine on 5-HT metabolism, two treatments were used.

Strychnine (5 × 10 ^{5} M) was first applied for 1 h. Strychnine by itself induced a marked increase in 5-HT metabolism. Both [^{3}H]5-HT synthesis (+38% and +24%) and release (+102% and +36%) were significantly enhanced in rostral and caudal raphe cells respectively. In a second set of experiments, 30 min after the onset of the strychnine treatment, glycine was co-administered with its antagonist. The glycine-induced inhibitory effect on both [^{3}H]5-HT synthesis and release was totally blocked by presence of strychnine (Fig. 2).

Effects of GABA on 5-HT metabolism

At 10 ^{6} M GABA was ineffective, 5-HT metabolism started to be affected at 10 ^{5} M and a significant decrease (−24% and −19%) was observed in [^{3}H]5-HT release in rostral and caudal raphe cells respectively (Table 2). Application of GABA (10 ^{4} M) induced a decrease in 5-HT metabolism. Both [^{3}H]5-HT synthesis and [^{3}H]5-HT release were significantly reduced (−42%, and −23%, respectively) in rostral

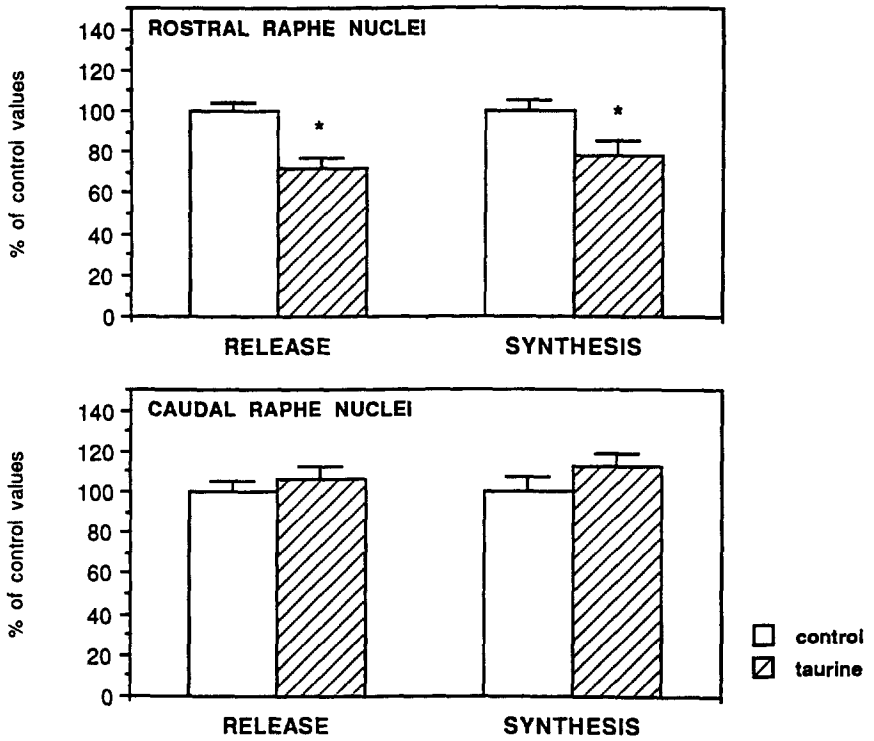

Fig. 1. Effects of taurine on 5-HT metabolism in rostral (B_4–B_9) and caudal (B_1–B_3) rhombencephalic raphe cells in primary cultures Rostral and caudal rhombencephalic raphe cells (12 day *in vitro*) were incubated with an artificial medium (CSF) containing L[^{3}H]tryptophan (10 μCi in 1 ml) and pargyline (5×10^{-5} M). After 60 min, the quantities of [^{3}H]5-HT synthesized in cells and released in medium were determined. Taurine (10^{-4} M) was added the last 30 min of incubation time. Data are the means ± SEM of results obtained from 12 samples. Data were statistically evaluated by Mann–Whitney test. *$P < 0.05$ when compared to control values.

raphe cells. In caudal raphe cells a significant decrease, was only observed in [^{3}H]5-HT release (−22%). No significant reductions (−18%) in [^{3}H]5-HT synthesis were detected (Table 2).

Table 2 Effects of GABA on 5-HT metabolism in rostral and caudal rhombencephalic raphe cells in primary cultures

	Rostral raphe nuclei		Caudal raphe nuclei	
	Release	Synthesis	Release	Synthesis
Control	100 ± 5	100 ± 7	100 ± 5	100 ± 6
GABA (10^{-6}M)	88 ± 6	92 ± 7	89 ± 7	96 ± 6
GABA (10^{-5}M)	76 ± 6(a)	86 ± 6	81 ± 6(a)	90 ± 8
GABA (10^{-4}M)	58 ± 5(a)	77 ± 6(a)	78 ± 6(a)	82 ± 7

Rostral and caudal rhombencephalic raphe cells (12 day *in vitro*) were incubated with an artificial CSF containing L[^{3}H]tryptophan and pargyline (5×10^{-5} M) for 60 min. GABA was added at various concentrations the last 30 min of incubation time Data were statistically evaluated by Mann–Whitney test

(a) $P < 0.05$ when compared to control values.

In order to determine the GABA receptor-subtypes involved in the inhibitory action of GABA on 5-HT metabolism, three paradigms were used.

First bicuculline (5×10^{-5} M) the $GABA_A$ receptor antagonist was applied for 1 h. Bicuculline alone induced a marked increase in 5-HT metabolism, both [^{3}H]5-HT synthesis (+31% and +31%) and [^{3}H]5-HT release (+ 147% and + 84%) were significantly enhanced in rostral and caudal raphe cells respectively (Fig. 3).

In a second set of experiments, 30 min after the onset of the bicuculline treatment, GABA (10^{-4} M) was co-administered with its antagonist. In presence of bicuculline, GABA application did not decrease 5-HT metabolism as compared to control values. But a significant decrease of 5-HT synthesis (−27%) and release (−23%) was again detected as compared to bicuculline treatment only in rostral raphe nuclei (Fig. 3).

Finally, baclofen, a $GABA_B$ receptor agonist was

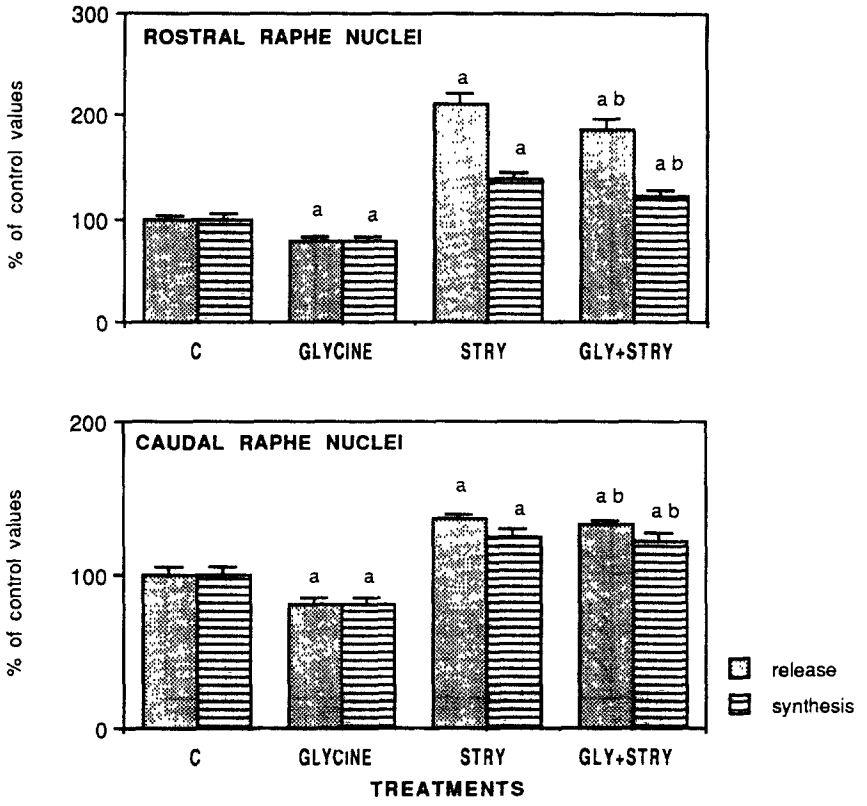

Fig. 2. Effects of glycine on 5-HT metabolism in rostral (B_4–B_9) and caudal (B_1 B_3) rhombencephalic raphe cells in primary cultures. Experiments were carried out as described in Fig. 1 except that glycine (Gly) (10^{-3} M) and strychnine (Str) (5×10^{-5} M) were used individually or in combination. Strychnine was applied for 60 min and glycine the last 30 min. Results are the means ± SEM of values obtained in 12 samples. Data were statistically evaluated by Mann–Whitney test. a, $P < 0.05$ when compared to control values. b, $P < 0.05$ when compared to glycine values.

applied for 30 min. Baclofen induced a significant decrease in [^{3}H]5-HT release (−28%), and synthesis (−23%) in rostral raphe cells and did not modify [^{3}H]5-HT metabolism in caudal raphe cells (Fig. 3).

Effects of GLU on 5-HT metabolism

At 10^{-5} and 10^{-4} M GLU enhanced 5-HT metabolism both in rostral and caudal raphe cell cultures. At 10^{-6} M GLU was ineffective (Table 3). To verify the specificity of the GLU-effect, PK 26124 (10^{-4} M), a glutamatergic transmission antagonist was added for 60 min alone or in co-administration with GLU (10^{-4} M). Attempts have also been made to characterize the GLU receptor subtypes involved in the GLU stimulating-effect in 5-HT metabolism. In this respect several agonists and/or antagonists of NMDA and/or quisqualate receptors were used.

On cultures of rostral rhombencephalon. PK 26124, by itself, significantly decreased both the [^{3}H]5-HT synthesis (−22%) and release (−20%) (Fig. 4). The GLU stimulating effect was entirely antagonized by the combined presence of PK 26124 (Fig. 4).

NMDA receptor subtypes. The NMDA (5×10^{-5} M) applied for 30 min stimulated 5-HT metabolism. [^{3}H]5-HT synthesis by +28% and [^{3}H]5-HT release by +69%. To verify the specificity of the NMDA stimulating-effect APV, a specific NMDA receptor antagonist, was used.

A decrease of 5-HT metabolism was induced by a 60

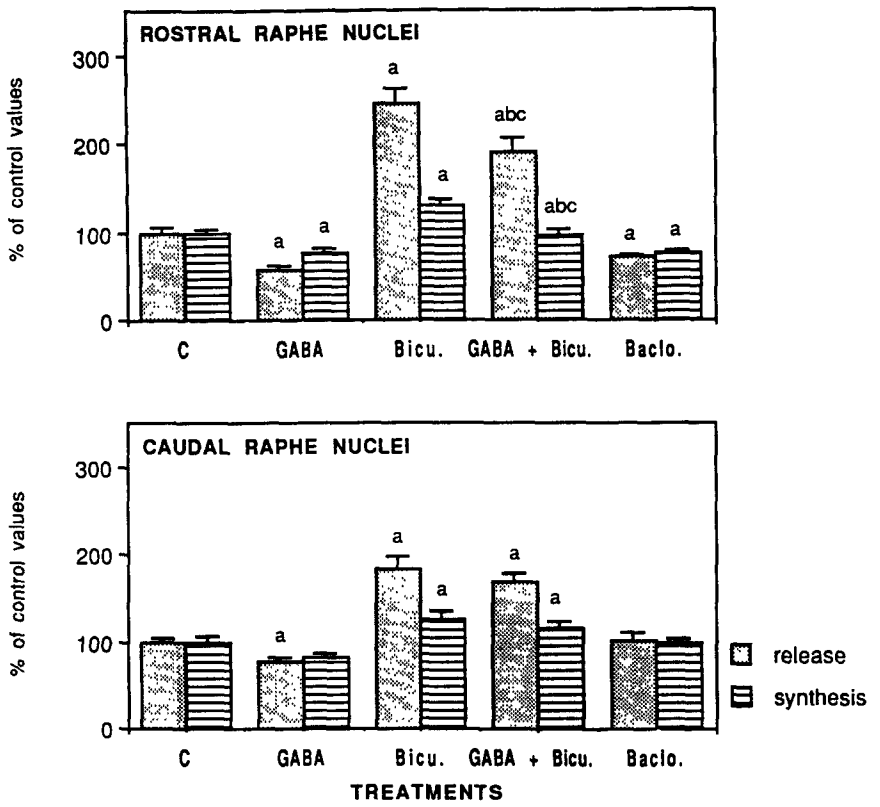

Fig. 3. Effects of GABA on 5-HT metabolism in rostral (B_4–B_9) and caudal (B_1–B_3) rhombencephalic raphe cells in primary cultures. GABA (10^{-4} M and (RS) -baclofen (baclo) (5×10^{-5} M) were added the last 30 min. of incubation time and bicuculline (Bic) (5×10^{-5} M) for 60 min. alone or in combination. Results are the means ± SEM of values obtained from 12 samples. Data were statistically evaluated by Mann–Whitney test. a, $P < 0.05$ when compared to control values. b, $P < 0.05$ when compared to GABA values. c, $P < 0.05$ when compared to bicuculline values.

min APV (5×10^{-5} M) treatment. [^{3}H]5-HT synthesis and release were equally affected (−25%). In a second set of experiments NMDA (5×10^{-5} M) was co-administered 30 min after the onset of the APV treatment. The stimulating effect of NMDA was antagonized by the presence of APV (Fig. 5).

Quisqualate receptor subtypes. Quisqualic acid (10^{-5} M) applied for 30 min in rostral rhombencephalon cells did not modify 5-HT metabolism. No effects were observed with AMPA (10^{-5} M) (Fig. 6).

On cultures of caudal rhombencephalon

The GLU stimulating effect was antagonized by PK 26124 (10^{-4} M). PK 26124 by itself did not significantly modify 5-HT metabolism (Fig. 4).

NMDA receptor subtypes. The NMDA (5×10^{-5} M) applied for 30 min stimulated 5-HT metabolism in caudal raphe cells (Fig. 5). Both [^{3}H]5-HT synthesis and release were increased, +28% and +62% respectively.

This effect was totally antagonized by presence of APV (5×10^{-5} M) in incubation medium. APV alone did not alter 5-HT metabolism (Fig. 5).

Quisqualate receptor subtypes. Quisqualic acid (10^{-5} M) applied for 30 min on caudal rhombencephalic cells increased [^{3}H]5-HT release by 30%. No changes in [^{3}H]5-HT synthesis were observed. Quisqualate mediates its effects through ionotropic and metabotropic receptor subtypes. In order to characterize the quisqualate receptor type involved in the quis-

Table 3 Effects of glutamate on 5-HT metabolism in rostral and caudal rhombencephalic raphe cells in primary cultures

	Rostral raphe nuclei		Caudal raphe nuclei	
	Release	Synthesis	Release	Synthesis
Control	100 ± 5	100 ± 6	100 ± 5	100 ± 7
Glutamate (10^{-6} M)	116 ± 8	108 ± 8	112 ± 7	96 ± 8
Glutamate (10^{-5} M)	148 ± 10(a)	123 ± 9(a)	128 ± 9(a)	118 ± 8
Glutamate (10^{-4} M)	193 ± 15(a)	149 ± 11	172 ± 12(a)	133 ± 9(a)

Rostral and caudal rhombencephalic raphe cells (12 day *in vitro*) were incubated with an artificial CSF containing L-[^{3}H]tryptophan and pargyline (5×10^{-5} M) for 60 min. Glutamate was added at various concentrations the last 30 min of incubation time. Data were statistically evaluated by Mann Whitney test

(a) $P < 0.05$ when compared to control values

qualic acid stimulating induced-effect the actions of AMPA and DNQX, ionotropic receptor agonist and antagonist respectively, were tested on 5-HT metabolism.

AMPA (10^{-5} M) applied for 30 min enhanced the [^{3}H]5-HT release (+36%). This stimulating effect was blocked by DNQX (5×10^{-5} M). DNQX alone did not modify [^{3}H]5-HT release (Fig. 6).

DISCUSSION

In mature CNS, 5-HT metabolism is controlled by other neuronal systems including amino acid neurotransmitters. This study was undertaken to further analyze whether such mechanisms are acting at an early stage of development. The regulations of the

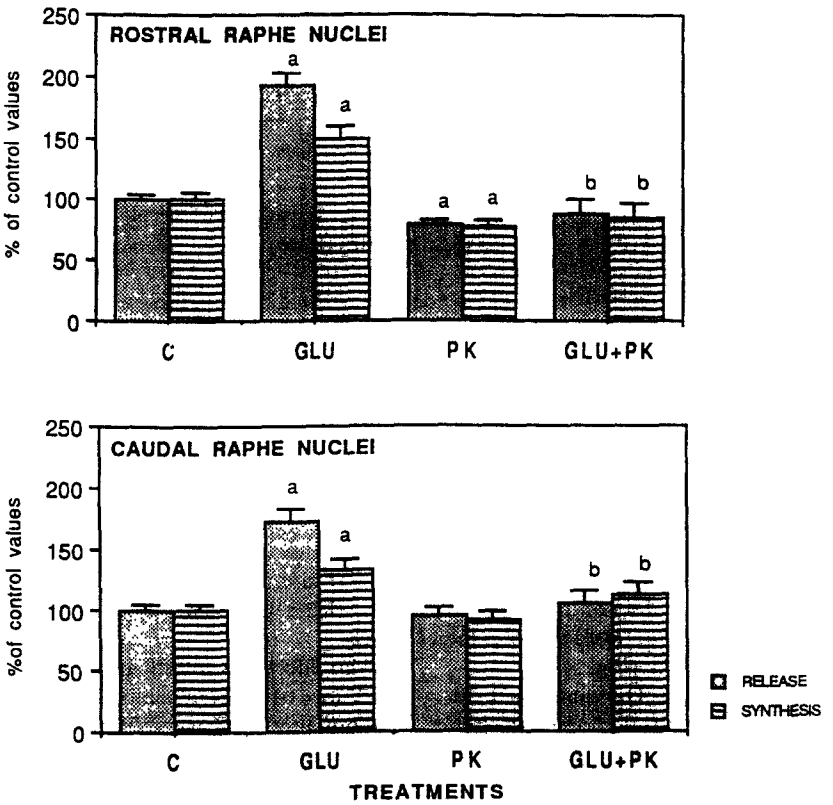

Fig. 4. Effects of glutamic acid on 5-HT metabolism in rostral (B_4–B_9) and caudal (B_1–B_3) rhombencephalic raphe cells in primary cultures. Glutamic acid (GLU) (10^{-4} M) was applied for 30 min and PK 26124 (PK) (10^{-4} M) for 60 min alone or in combination. Results are the means ± SEM of values obtained from 12 samples. Data were statistically evaluated by Mann–Whitney test a, $P < 0.05$ when compared to control values. b, $P < 0.05$ when compared to glutamate values.

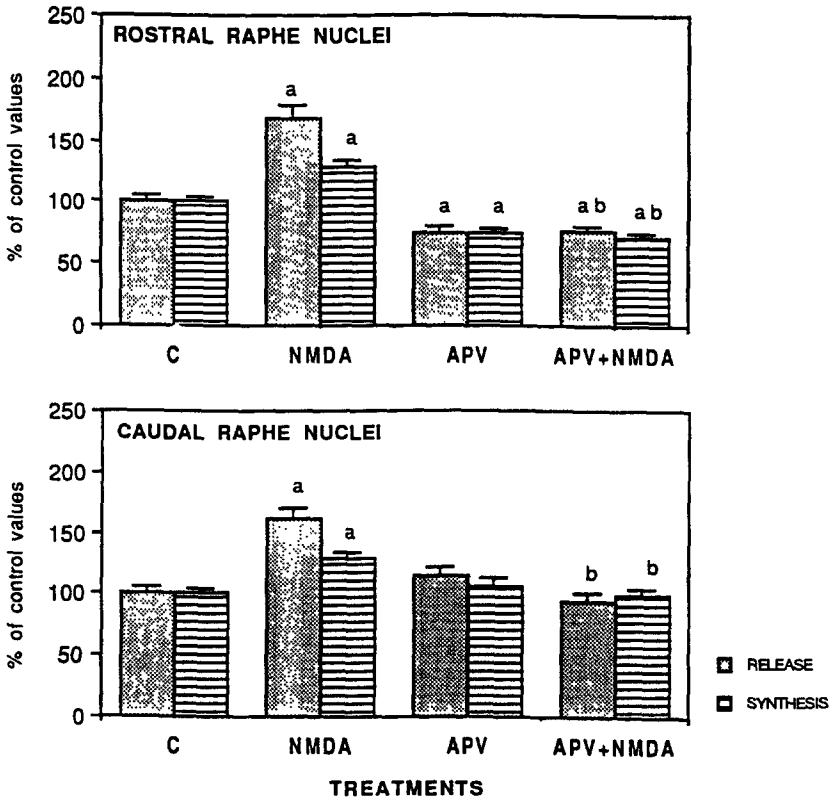

Fig. 5. Effect of *N*-methyl-D-aspartic acid on 5-HT metabolism in rostral (B_4–B_9) and caudal (B_1–B_3) rhombencephalic raphe cells in primary cultures. NMDA (5×10^{-5} M) was added in the medium for the last 30 min of the 60 min incubation time and APV (5×10^{-5} M) during 60 min. Drugs were, applied alone or in combination. Results are the means ± SEM of data obtained from 12 samples. Data were statistically evaluated by Mann–Whitney test. a, $P < 0.05$ when compared to control values. b, $P < 0.05$ when compared to NMDA values.

5-HT metabolism were studied in 12 day *in vitro* cultures of rostral (B4–B9) and caudal (B1–B3) raphe nuclei performed in a serum-free synthetic medium (Bottenstein and Sato, 1979). A specific and sensitive biochemical isotopic method was used to measure 5-HT synthesis and release. It consisted in measuring endogenously synthesized [^{3}H]5-HT from [^{3}H]TRP having a high specific activity (Hery *et al.*, 1979). Since tryptophan hydroxylase is selectively located in 5-HT neurons, we are certain that the newly formed [^{3}H]5-HT originates from serotoninergic neurons and not from cells possessing only uptake mechanisms such as the APUD cells.

The present report further describes that 5-HT metabolism is controlled by amino acid transmitters in rhombencephalic primary cell cultures. The 5-HT metabolism is decreased by taurine, glycine and GABA whereas an increase was induced by GLU. Attempts were made to determine the presence and to quantify the amounts of these amino acids in cells and incubation medium. GABA, glycine, GLU and taurine have been detected in a millimolar range in cells and in a micromolar range in the medium after a 60 min incubation period.

In CNS the amino acid transmitters are present simultaneously in glial and in neuronal cells. Astrocytic glial cells represent an important inactivation system principally for GLU and GABA. Glial cells can accumulate and release GLU, GABA, glycine and taurine similarly to neurons although the contribution of newly synthesized glial glutamate may be small in comparison to that of neuronal origin, (Hamberger *et al.*, 1981; Huxtable, 1989 for review). As the rhombencephalic primary cell cultures were grown in synthetic serum-free medium less than 5% of glial cells were present in primary cultures in our experimental

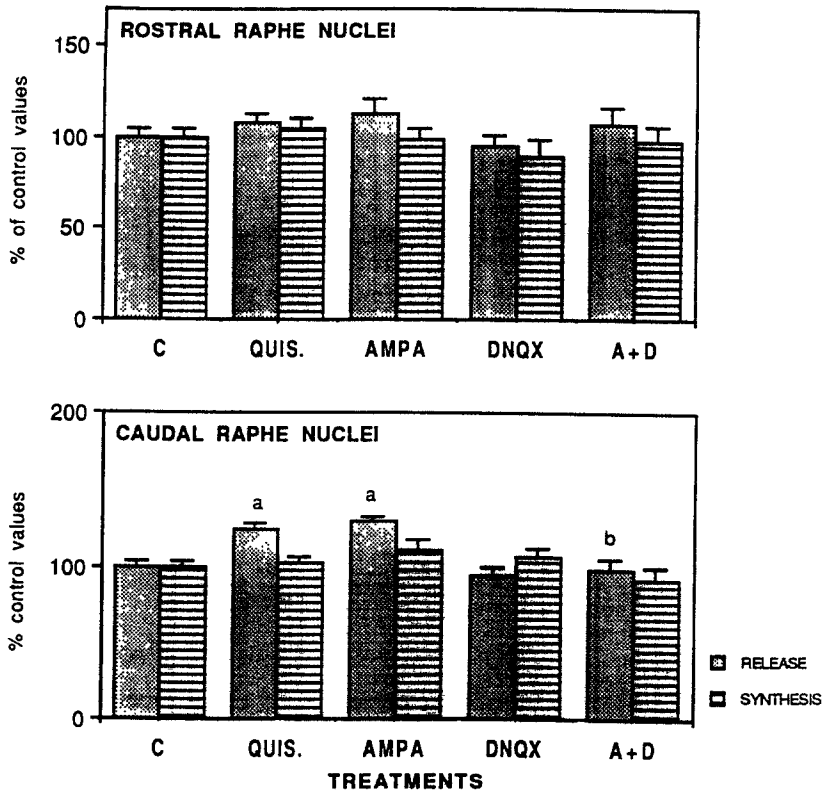

Fig. 6. Effects of quisqualic acid and AMPA on 5-HT metabolism in rostral (B_4–B_9) and caudal (B_1–B_3) rhombencephalic raphe cells in primary cultures. Quisqualic acid (QUIS.) (10^{-5} M) and AMPA (A) (10^{-5} M) were applied for 30 min and DNQX (D) (5×10^{-5} M) for 60 min. Compounds were used alone or in combination. Results are the means ± SEM of data obtained from 12 samples. Data were statistically evaluated by Mann Whitney test. a, $P < 0.05$ when compared to control values. b, $P < 0.05$ when compared to AMPA values.

conditions as reported previously by using immunostaining with GFAP antibodies (Pin *et al.*, 1986; Giraud *et al.*, 1991). This suggests that GABA, GLU, glycine and taurine are mainly located in neuronal cells.

Taurine

The discovery of high concentrations of taurine in brain led to speculate that taurine might play a neurotransmitter role in brain and spinal neurons (Huxtable, 1989). Taurine is one of the most abundant free amino acids in the mature brain. It is noteworthy that taurine was also the most abundant amino acid found in rostral as well as caudal rhombencephalon cell cultures. There are 20 times more taurine than GABA, 10 times more than glycine and 6 times more than GLU in rostral raphe cells. About 10% of the taurine cell content was released in 60 min in rostral as well as caudal raphe cells. In mature CNS, taurine is released and, by the use of the microdialysis, micromolar extracellular taurine concentrations were estimated in the dentate gyrus (Lerma *et al.*, 1986). Moreover, taurine release is enhanced by NMDA (Lehmann *et al.*, 1985). Known for its function as an osmoregulator in brain (Huxtable 1989), it is now generally accepted that in both central and peripheral nervous system, taurine modulates membrane excitability by decreasing the concentration of intracellular free Ca^{2+} (Pasantes-Morales and Gamboa, 1980) and then inhibits the release of neurotransmitters (Kuriyama *et al.*, 1978). In agreement with this data, taurine application in rostral raphe cultures induced a decrease in both 5-HT release and synthesis. However, the mechanism of action of taurine might be more complicated. A role for taurine as a modulator of the GABA-benzodiazepine receptor complex was sug-

gested (Medina and De Robertis, 1984) and could explain the taurine effects. Indeed, a GABA-induced decrease of 5-HT metabolism was also observed. Unfortunately, no specific taurine receptor antagonists were available and this did not allow us to verify whether taurine acts directly via taurine receptors. Nevertheless, taurine induced-effect on 5-HT metabolism was only detected in rostral raphe nuclei culture, although a GABA-induced decrease in 5-HT metabolism was detected in both rostral and caudal rhombencephalic cells. This stresses out the direct action of taurine on 5-HT metabolism.

Glycine

Glycine is found in all tissues, and its presence *per se* in a brain area does not infer a neurotransmitter role in that area. Nevertheless, distribution studies do offer valuable clues. Indeed, glycine concentrations found in spinal cord, medulla and pons are much higher than those in other CNS regions (Aprison *et al.*, 1969). In our study, relatively high levels of glycine in both rostral and caudal rhombencephalon in culture were found, suggesting that in these culture conditions glycinergic neurons were able to grow. Moreover, the ratio of medium vs cell amounts indicative of a high level of glycine release emphasized the functional role of glycine in primary cultures.

Relatively few specific pharmacological agents were found to interfere with glycine neuronal systems. The postsynaptic glycine receptor is a ligand-gated chloride channel mediating strychnine-sensitive inhibition of neuronal activity in different brain structures (Curtis *et al.*, 1968; Young and Snyder, 1973; Betz and Becker, 1988).

The glycine receptor has been purified from mammalian spinal cord and represents a pentamer composed by 4 subunits: α_1 α_2 α_3 and β (Betz, 1990) with a significant sequence homology with nicotinic and $GABA_A$ receptor proteins (Grenningloh *et al.*, 1990a). Moreover, α subunit variants have been identified which are thought to represent ligand-binding subunits (Grenningloh *et al.*, 1990b). During development, α_2 and β subunits were expressed in rat brain at early embryonic stages (E 14) (Malosio *et al.*, 1991). Thus, the expression of α_2 subunit in rhombencephalon correlates with the presence, during embryogenic stages, of glycine receptor protein which binds strychnine (Becker *et al.*, 1988).

We report here a significant decrease of 5-HT synthesis and release by glycine application. This glycine-induced inhibition was blocked by the alkaloid strychnine. These results fit well with the general inhibitory action of glycine in the lower brainstem, mediated through an increase in chloride ion conductance. Furthermore, strychnine by itself induced a marked increase in 5-HT release and synthesis, indicating the presence of a massive and tonic inhibitory control on 5-HT release by the glycinergic systems. The presence of large amounts of glycine released in the incubating medium explains why high concentrations of glycine have to be used to induce a decrease in 5-HT metabolism.

GABA

The failure to obtain any major changes in glutamate decarboxylase activity (GAD) in the dorsal raphe nucleus after lesions of its afferent pathways (Belin *et al.*, 1979) and the decrease in GAD after local kainic acid injections (Ottersen and Storm-Mathisen, 1984) strongly suggest that local GABA neurons contribute to the high GAD content in this structure (Massari *et al.*, 1976). Consistent with this observation were the levels of GABA found in rostral rhombencephalon cultures. High levels of GABA were also measured in caudal rhombencephalon.

GABA decreased [^{3}H]5-HT release and synthesis in both rostral and caudal rhombencephalon cultures. The results observed in rostral cultures are comparable to that observed in mature brain both in target area of 5-HT neurons, like caudate nucleus (Becquet *et al.*, 1988), and in the structure of nerve cell bodies i.e. dorsal raphe nucleus (Becquet *et al.*, 1990a). However a difference existed between GABA regulation of 5-HT at the axon terminal level vs nerve cell body level. In the first case, bicuculline administration by itself was without effect on spontaneous [^{3}H]5-HT release, indicating that GABA control was phasic in nature, contrariwise to that observed at the cell body level where $GABA_A$ antagonist, induced a marked increase in [^{3}H]5-HT release (Becquet *et al.*, 1990a). In primary cultures of raphe nuclei, bicuculline by itself induced a marked increase in [^{3}H]5-HT release, indicating that GABA control on 5-HT metabolism was tonic in nature.

The main difference in GABA control of 5-HT metabolism between rostral and caudal rhombencephalon cultures resulted in the receptor-types involved. The action of GABA can be mediated by $GABA_A$ and $GABA_B$ receptors (Bowery *et al.*, 1987). In rostral rhombencephalon cultures contrariwise to caudal ones, bicuculline was not sufficient to entirely block the GABA induced effect on 5-HT metabolism. Furthermore, the $GABA_B$ agonist baclofen also decreased 5-HT release. Thus, it appears that $GABA_A$ and $GABA_B$ receptors are involved in the control of 5-HT metabolism in rostral rhombencephalon cultures

whereas only $GABA_A$ receptors are involved in the control of 5-HT in caudal rhombencephalon.

No direct data are available about the presence of GABA receptors during early stages of development. Only regional development of benzodiazepine-binding sites in fetal brain was reported. Benzodiazepine binding sites are associated with $GABA_A$ receptor. Autoradiographic studies revealed that central benzodiazepine binding sites were developed in a distinct pattern with a general caudo-rostral gradient. Specific binding was first detected at gestational day 14 in the spinal cord and lower brainstem (Schlumpf *et al.*, 1983). Electrophysiological studies performed in postnatal period indicate that the $GABA_A$ response obtained in dorsalis raphe nucleus cells from neonate rats were similar to those associated with the $GABA_A$ receptor subtype. Moreover, pharmacological approaches suggest that some of the GABA inhibitory actions are mediated by the $GABA_B$ receptors and that their functional effects do not appear to vary with age (Smith and Gallager, 1987).

Glutamic acid

In both types of culture GLU induced a stimulation in 5-HT metabolism. The specificity of the stimulatory effect of GLU on [^{3}H]5-HT metabolism was first studied using PK 26124 as a broader spectrum antagonist of glutamatergic transmission. The mechanisms by which glutamatergic transmission is blocked by PK 26124 are not yet clearly determined. However, results from numerous *in vivo* and/or *in vitro* experiments indicate that glutamic acid-induced effects are antagonized by this compound (Benavides *et al.*, 1985; Cheramy *et al.*, 1986; Becquet *et al.*, 1990b). In this study, GLU control of 5-HT metabolism was prevented by PK 26124 in both rostral and caudal rhombencephalic cell cultures. But data obtained with PK26124 did not allow the precise determination of the glutamatergic receptor type involved in the regulation of 5-HT metabolism.

The excitatory responses to glutamate are mediated by several receptor subtypes commonly classified as NMDA and non-NMDA glutamate receptor types based on their preferential responses to synthetic EAA receptor agonists (McLennan, 1983; Watkins *et al.*, 1990). Non-NMDA receptors are divided into separate quisqualate and kainate subtypes on the basis of their differential sensitivity to agonists. However, binding studies show that quisqualate is not a specific agonist for only one type of receptor. This gives rise to the concept of two subtypes of quisqualate receptor, one referred to as AMPA receptor (Monaghan *et al.*, 1989) also called quisqualate "ionotropic receptor" and the second, coupled to inositol phosphate, is termed "metabotropic receptor" (Sladeczek *et al.*, 1985; Recasens *et al.*, 1988).

In the present report, the GLU-induced stimulation of 5-HT metabolism was mimicked by NMDA in both rostral and caudal rhombencephalic cell cultures. Furthermore, the NMDA-stimulating effect was totally prevented by the presence of APV, a specific NMDA receptor antagonist (Olverman *et al.*, 1988). In addition, a decrease in [^{3}H]5-HT release and synthesis was induced by APV alone in rostral rhombencephalic cell cultures. It is noteworthy that APV did not decrease 5-HT metabolism in caudal rhombencephalon cell cultures. Similar data were observed with PK 26124. These results show that glutamatergic control of 5-HT metabolism is tonic in nature in rostral and phasic in caudal rhombencephalic cell cultures. Although, on one hand similar amounts of endogenous GLU in cells and CSF were present in both types of cultures and on the other hand, the release of GLU was greater in caudal (50%) than in rostral (27%) rhombencephalic cell cultures. The tonic nature of glutamatergic control of 5-HT metabolism could be due to an indirect action of GLU on serotoninergic neurons via interneuronal systems. We recently reported that, in cat caudate nucleus in *in vivo* experiments, the 5-HT release was tonically controlled by GLU and NMDA, through GABAergic neurons (Becquet *et al.*, 1990b).

Quisqualate induced an increase in [^{3}H]5-HT release from caudal rhombencephalon cells and it was ineffective in 5-HT metabolism in rostral rhombencephalon cell cultures. A similar increase in [^{3}H]5-HT release was obtained with AMPA and this effect was blocked by DNQX indicating that the quisqualate stimulating induced effect was obtained via the quisqualate "ionotropic receptor". It is noteworthy that (1) [^{3}H]5-HT synthesis was not modified unlike that observed with GLU and NMDA respectively (2) the quisqualate control was phasic in nature and finally (3) the effect of quisqualate was observed only in caudal and not in rostral rhombencephalic cell cultures.

No data were available on the identification and quantification of EAA receptors during embryonic stages. Presence of EAA receptors in neuronal primary cultures suggested a role during brain development. Thus, a rapidly growing body of evidence suggested that EAA were involved in a variety of physiological processes during development. The role of GLU is particularly known for its neurotoxic activity. The EC_{50} of GLU neurotoxicity in culture of spinal cord was 100–200 μM (Regan and Choi, 1991).

concentrations much greater than those measured in the media of both types of rhombencephalic cultures. Overstimulation of NMDA receptors was implicated in several *in vivo* and *in vitro* models in neuronal death (McDonald and Johnston, 1990). AMPA induced a pattern of degeneration that markedly differed from the one elicited by NMDA (Garthwaite and Garthwaite, 1991). In contrast to their suspected neuropathological involvement, NMDA receptors appear to play a role in regulating neuronal cell survival during maturation. Thus, high doses of NMDA receptor antagonists induce neuronal cell death in developing spinal cord cultures whereas low concentrations increased neuronal survival (Brenneman *et al.*, 1990). This influence was stage-dependent, older cells being more sensitive to NMDA induced neurotoxicity than younger ones (Peterson *et al.*, 1989). In cultured neurons, NMDA promotes the stabilization of active synapses, neuritic outgrowth and dendritic branching (Collingridge and Singer, 1990).

In conclusion, our data show that [^{3}H]5-HT release and synthesis is controlled by inhibitory amino acid neurotransmitters, taurine, glycine and GABA and by EAA via NMDA and quisqualate "ionotropic" receptors in the rostral and caudal rhombencephalic cell cultures. This study demonstrates that 5-HT metabolism is controlled by amino acid neurotransmitters during early stages of development similarly to that reported in mature brain. In immature brain all these neurotransmitters act as developmental signals or regulators. It has already been shown that GABA, taurine, glycine and EAA (Huxtable, 1989; Ruzicka and Jhamandas, 1993) and now 5-HT are interacting to control their metabolism. Thus, the role of these neurotransmitters on brain development becomes more complex particularly to determine whether the observed effects are direct or indirect.

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