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Isolation and Identification of Two Learning-Induced Brain Peptides

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TATE, D. F., L. GALVAN AND G. UNGAR. *Isolation and identification of two learning-induced brain peptides.* PHARMAC. BIOCHEM. BEHAV. 5(4) 441–448, 1976. – Goldfish were trained either to avoid the blue compartment of a tank and swim into the green compartment or, conversely, to avoid green and prefer blue. Preliminary experiments indicated that acquisition of the avoidance behavior was associated with the presence in the brain of two peptides, one found in blue avoiding (BA), another in green avoiding (GA) fish. With the help of behavioral bioassays, the peptides were isolated and purified, and their structure was determined by ultramicroanalytical techniques. The sequence of the BA peptide, pglu-ile-gly-ala-val- phe-pro-leu-lys-tyr-gly-ser-lys-OH was reproduced by synthesis. Sequential analysis of the GA peptide gave two alternative structures, NAc-lys-gly-gln-ile-ala-val-phe-pro-leu-lys-tyr-gly-ser-OH or NAc-lys-gly-ala-val-gln-ile-phe-pro-lys-tyr-gyl-ser-OH, both of which are being synthetized to be compared with the natural compound. Overlapping sequences between the BA and GA peptides suggest the existence of a family of peptides associated with behavior based on color discrimination.

Avoidance Learning Goldfish Peptides Memory Color discrimination Chromodiopsins

FOR the last two decades, evidence had been accumulating for a decisive role of peptides in the control of behavior. Hypothalamic releasing and release-inhibiting hormones have been shown to be involved in innate behavior motivated by organic drives [30]. Peptide hormones and their derivatives have also been implicated in the storage of acquired information [6]. Furthermore, formation of peptides in brain has been associated with the acquisition of new behavior [6, 29–31]. The structure of two of these learning-induced peptides has been determined and reproduced by synthesis [4, 14, 34]. Isolation of these substances was accomplished under the guidance of the controversial behavioral bioassay method but, more recently, their formation in the brain was confirmed by chemical analysis [28,33].

Zippel and Domagk [38] showed that fish trained to prefer green to red (by being fed in the green compartment of a divided tank) develop in their brain a substance that, injected into untrained fish, induces preference for the green. Ungar *et al.* [35] have modified the experimental conditions and used electric shock as negative reinforcement to train animals to avoid one color and prefer another color presented simultaneously. The present paper deals with the purification and structural analysis of two peptides formed in the brain of goldfish trained for avoidance behavior based on color discrimination.

METHOD

Animals and Procedures

Behavioral training. Goldfish (*Carassius auratus*) 5 to 8 cm long, obtained from Ozark Fisheries, Stoutland, Missouri, were trained either to avoid the blue compartment of a tank and swim to the side lighted in green or to avoid the green and take refuge in the blue [35]. Simultaneous training of a large number of fish was done in 16 eight gallon plastic tanks. Each tank was provided with a metal partition dividing it into two compartments, one lighted in blue (Eastman Kodak filter No. 47, 370–510 nm), the other in green (No. 48, 486–630 nm). The partitions left a clearance of 4.5 cm at the bottom of the tank to allow the fish to swim from one side to the other. The compartments were lighted from the sides of the tanks, the filters being adjusted to provide an output of equal intensity for the two colors. The two ends of the tanks were fitted with wire-mesh electrodes so that current flowed between them and the partition. Further details were given in a previous publication [35].

The training sessions were programmed so that blue light went on in one compartment the same time as green light appeared on the opposite side. The colors were presented in random order (Gellerman sequence). After 20 sec of light presentation, while the lights stayed on, electric shocks

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(equal polarity square waves, 2 mA of 50 msec duration every 2 sec) were turned on for 10 sec on the side of the color to be avoided. After an intertrial dark interval of 30 sec, the cycle started again to reach 15 light and shock presentations per day. Training was terminated when 25 out of the 30 fish contained in one tank made 13 out of 15 correct responses, i.e., swam away from the color to be avoided or stayed on the side of the color to be preferred. This criterion was reached in 12 to 14 days. The validity of the mass training procedure was ascertained in preliminary experiments by individual testing of the animals having satisfied the group criterion. The tests showed that 80 to 90% of the animals gave the correct responses. Although this may have caused a dilution of the active material, mass training was the only way to collect the necessary amount of material within a realistic period of time.

In all, 8,400 fish were trained to avoid the blue and prefer the green (BA and 8,300 fish were trained to the opposite behavior (GA). Twenty-four hours after the criterion was reached, the brains were removed, placed on dry ice and kept at −20° C until extraction. Collection of the material took about 18 months.

Behavioral bioassay. The test used during purification for selecting the fractions containing the active material were done under conditions similar to those of the training. There was, however, no electric shock applied at any time and the fish were tested individually. Each animal received five 30 sec presentations of blue light and five of green light on the side of the tank where it happened to be. Avoidance response consisted in the fish swimming to the opposite side of the tank during the 30 sec of light presentation. The first test was done before injection and, according to its results, the fish were divided into groups of 8, each group having approximately equal mean avoidances. The brain extracts or their fractions were injected intracranially in 40 μl volumes. Control groups were injected either with identically prepared extracts of untrained goldfish brain or, in the case of purified fractions, with the appropriate vehicle. All testing was done under blind conditions.

Evaluation of the activity of crude brain extracts was done by comparing the mean percent blue avoidances with the mean percent green avoidances and assessing the significance of the difference between them by means of Student's *t*-test. Comparisons between recipients of brain material from blue-avoiding, green-avoiding and control donors were also made by analysis of variance and Duncan's range test [36]. During purification procedures, the behavioral activity of the various fractions was expressed in terms of mean net avoidance (blue avoidance−green avoidance) over two days of testing. Significance of the differences between pre- and postinjection net avoidances was calculated by the method of paired comparisons [11].

Chemical procedures. Frozen brains were homogenized and partitioned by the cold phenol method [9]. Since previous studies had shown that the active material was complexed with RNA [35], the aqueous (RNA-containing) fraction was dialyzed against 20 volumes of 0.05 M ammonium acetate buffer at pH 3.7 for 48 hours at 4° C. After lyophilization, the dialyzate was passed through a refrigerated column (2.4 cm × 88 cm) of Sephadex G-25 fine (Pharmacia Fine Chemicals). After elution with pyridine-acetic acid buffer, pH 6.5, at a flow rate of 35 ml/hour, the fractions, redissolved in small volumes of distilled water, were injected into goldfish and tested for behavioral activity as described above.

Further purification of the fractions containing the active material was done by thin-layer chromatography on Polygram cellulose MN-300 plates (Brinkmann Instr.) with the following solvent systems: n-butanol:glacial acetic acid:water; 4:1:1 (solvent I); amyl alcohol:pyridine:water; 35:35:30 (solvent II) and n-propanol:30% NH_4OH; 67:33 (solvent III). At each separation, the active fraction was determined by bioassays of the eluates of the cellulose scrapings. A small sample was also sprayed with fluorescamine (Roche Products).

The active fractions were then passed through a Sephadex G-25 superfine column (0.6 cm × 30 cm) to remove cellulose and other contaminants. The columns were eluted with pyridine-acetic acid buffer at pH 3.9 at a flow rate of 3 ml/hour. In some cases, the fraction was further purified on a Brinkmann FFI free-flow electrophoresis apparatus, under the following conditions; separation buffer, 0.5 N acetic acid, pH 2.4; electrode rinse buffer, 1.0 N acetic acid; buffer pump, 6; dosing pump, 24; emptying time, 20 min; port number, 4; 2300 V; 5° C. The procedure yields 48 fractions: glutamic acid elutes in fraction 19 and lysine in fraction 47. Both of the peptides under study were found in alkaline fractions.

Purity of the isolated material was verified by dansylation [10,23]. A pure peptide, when fully dansylated, should have only one dansyl derivative. Furthermore, if the peptide is hydrolyzed after dansylation, it should yield only one N-terminal amino acid. Small samples of the isolated product were made to react with 1 μl aliquots of a solution of dansyl chloride (dimethylaminonaphthalenesulfonyl chloride (0.1% in acetone) and 3 μl of 0.05 M solution of sodium bicarbonate (pH 10) [3, 14, 21]. The reaction was run for 3 hours in the dark, after which time the mixture was lyophilized and redissolved, usually in 1 μl of 50% pyridine in water. All the steps of the reaction were carried out in 100 mm long and 1.6 mm dia. flame sealed capillaries (Kimble).

The dansyl derivative was submitted to two dimensional thin-layer chromatography on polyamide plates (Gallard-Schlesinger) as described by Neuhoff *et al.* [21] and Burzynski [3,4]. The following solvent systems were used for developing the plates: formic acid:water, 1.5:100 (designated as 1°I) for the first dimension, and heptane: butanol; acetic acid, 3:3:1 (2°II) or benzene:acetic acid, 14:1 (2°III) for the second dimension. The plates were examined and photographed under short-wave ultraviolet light (Mineralight C-81 from Ultra Violet Prod. Inc.).

Amino acid analyses were done after acid hydrolysis. Samples dissolved in 1 μl of 6.7 N HCl were heated to 105° C for 24 hr in sealed capillary tubes. The hydrolyzates were dansylated and chromatographed as described above. Amino acids were identified by comparison with maps of their dansyl derivatives and confirmed by co-chromatography with standard dansyl amino acids (Calbiochem).

To eliminate the possibility of acid sensitive residues, complete digestion was also done with aminopeptidase M (3.4.1.2: referred to below as AM), obtained from Henly & Co., New York (5000 mU/mg). The enzyme was dissolved in 0.1 M sodium borate and 0.01 M $CaCl_2$ at 0.2 μg/μl and 1 μl of the solution was mixed with amounts of peptides equivalent to 5 to 20 g of brain. Complete hydrolysis required incubation for 24 hr at 17° C. The amino acids were identified by dansylation and chromatography as described above.

Quantitative amino acid analysis was carried out by

TABLE 1

BEHAVIORAL ACTION OF BRAIN EXTRACTS FROM BLUE AVOIDING (BA), GREEN AVOIDING (GA) AND UNTRAINED (C) FISH

From	BA fish					GA fish					C fish				
	BA*		GA†			BA*		GA†			BA*		GA†		
Days	%	± S.D.	%	± S.D.	p‡	%	± S.D.	%	± S.D.	p‡	%	± S.D.	%	± S.D.	p‡
0	22.5	25.9	15.0	22.3	NS	26.0	26.2	19.0	22.4	NS	22.5	20.6	18.3	22.8	NS
1	46.0	34.6	17.5	20.7	<0.01	23.0	22.6	42.0	28.2	<0.02	23.2	21.0	15.8	22.0	NS
2	57.5	36.0	16.0	20.4	<0.001	25.0	26.7	45.0	27.8	<0.02	18.3	31.6	23.4	21.8	NS
3	56.0	29.1	14.0	15.3	<0.001	38.0	30.4	47.5	28.1	NS	20.8	17.2	15.0	17.0	NS
4	52.5	36.0	25.0	28.5	<0.05	34.0	30.7	56.0	33.2	NS	28.8	26.2	22.5	21.8	NS

Pooled results of three experiments, each involving 8 fish injected with BA, GA or C (100 mg brain equivalent per fish). On the fourth day only two groups of fish were tested.

*blue avoidances; †green avoidances; ‡significance of the difference between percent blue and green avoidances, computed by *t*-test (one-tailed).

TABLE 2

ANALYSIS OF VARIANCE (REPEATED MEASURES) OF NET AVOIDANCES IN FISH INJECTED WITH BRAIN EXTRACTS FROM BA, GA, AND C DONORS (FROM DATA SUMMARIZED IN TABLE 1)

Source of variation	SS	*df*	MS	F	*p*
Donor training	251.45	2	125.73	27.79	<0.001
Animals within groups	291.21	69	4.22		
Days	7.90	2	3.95	1.51	NS
Donor training x days	9.43	4	2.36	0.90	NS
Days x animals within groups	360.67	138	2.61		

Computed with the net avoidance (BA-GA) measurements done on Days 1–3.

densitometry of the dansyl derivatives displayed on thin-layer plates and photographed with an overhead Polaroid MP-3 camera with type 46L film. Details of the procedures have been described by Burzynski [3].

The following enzymes were used in the sequential analysis of the peptides: Carboxypeptidase B (3.4.2.2; CPB), 135 units/mg, purchased from Sigma. It was dissolved at 0.01 μg/μl in 0.2 M N-ethylmorpholine buffer at pH 8.5 [2]. Trypsin (3.4.4.4), twice recrystallized, salt free, and bovine pancreatic α-chymotrypsin (3.4.4.5) were obtained from Schwarz-Mann. They were dissolved in 0.046 M tris and 0.012 M $CaCl_2$ at pH 8.1 [13] at concentrations ranging from 0.05 to 0.2 μg/μl and incubation times varying from 5 to 24 hr. Bovine cathepsin C (3.4.4.9) or dipeptidyl-aminopeptidase I (13.7 U/mg from Sigma) splits dipeptides from free N-terminal peptide substrates [5]. It was dissolved in a pH 5 buffer composed of 16 mM HCl, 0.8% pyridine, 52 mM aceitc acid, 14 mM 2-mercaptoethanol and 0.5 mM EDTA [15]. Concentrations varied from 0.1 to 2 μg/μl and incubation time from 12 to 72 hr.

The activity of all anzymes used in the sequential analysis of our peptides was first checked on synthetic substrates: Substance P and luteinizing hormone-releasing-factor (LRH) from Beckman Instr. and angiotensin II and lys-bradykinin from Schwarz-Mann.

RESULTS

Purification of the BA and GA Peptides

A total of 750 g of brain from fish trained for blue avoidance and 740 g of brain from green avoidance trained fish were extracted by the cold phenol method. The yield of crude RNA was 3 to 4 mg per g of brain.

Batches of the material were tested for behavioral activity. Fish were injected and tested as described above. The results summarized in Table 1 show that fish treated with material extracted from blue-avoiding donors made significantly more blue avoidances and less green avoidances and, conversely, recipients of green avoidance extracts made significantly more green avoidances and less blue avoidances than the controls injected with brain extracts from untrained donors. An analysis of variance of the repeated measures of net avoidance (BA-GA) taken during Days 1 to 3 in the 3 groups of animals, summarized in Table 1, showed that the only significant source of variation was the training given to the donor animals (Table 2). Application of Duncan's range test confirmed that all 3 groups were significantly different from the two other groups.

Table 3 outlines the steps in the purification of the peptides: dialysis, gel filtration of the dialyzate and thin-layer chromatography on cellulose plates. At each of the steps, the active fraction was located by bioassay but, because of the limited amount of material available, no quantitative estimations were made. Separation of the BA and GA peptides was made on thin-layer chromatography. After successive separation by three solvent systems, the peptides were further purified by gel filtration and the GA peptide by free-flow electrophoresis. Tables 4 and 5 show the results of the final test of the purified products and the adjacent fractions. The dansyl derivative of the purified BA peptide was chromatographed and showed a single spot of R_f 0.41 in solvent system 2° II. It remained at the origin when developed in the two other systems. After acid

TABLE 3

SUMMARY OF PURIFICATION STEPS (FOR DETAILS, SEE TEXT)

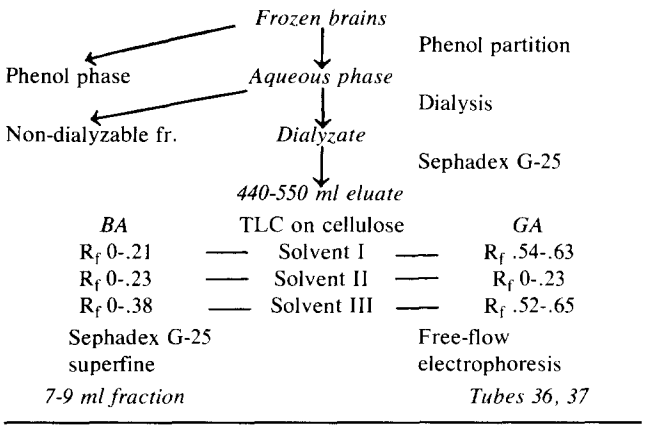

TABLE 4

BEHAVIORAL EFFECT OF PURIFIED BA PEPTIDE

Fraction*	Net Avoidance (BA-GA) ± S.D. Before Injection	After Injection†	p
6-7 ml	−15.0 ± 29.7	−8.75 ± 38.7	NS
7-8 ml	11.4 ± 32.3	54.3 ± 29.3	<0.025
8-9 ml	15.0 ± 33.3	40.0 ± 16.9	<0.05

Groups of 8 fish for each fraction.
*Fractions represent effluents collected from gel filtration on Sephadex G-25 superfine, short columns (for details see Method).
†Mean values of net avoidances 1 and 2 days after injection.

TABLE 5

BEHAVIORAL EFFECT OF PURIFIED GA PEPTIDE

Fraction*	Net Avoidance (BA-GA) ± S.D. Before Injection	After Injection†	p
34-35	23.0 ± 21.3	8.55 ± 19.5	NS
36-37	22.5 ± 16.6	−11.25 ± 13.6	<0.001
38-39	22.52 ± 22.5	11.25 ± 20.3	NS

Groups of 8 fish for each fraction.
*Fractions are tube numbers of effluents collected by free flow electrophoresis under conditions specified in the text (see Method).
†Mean values of net avoidance 1 and 2 days after injection.

hydrolysis the spot disappeared and was replaced by monodansyl-lysine, suggesting that the N-terminal was blocked and the peptide contained one or more lysines dansylated on the side chain. When the peptide was treated with 1 N NaOH for 18 hr at 4° C, glutamic acid appeared, indicating that the N-terminal group was pyroglutamic acid.

The GA peptide was not quite pure after passage on the Sephadex G-25 superfine column. After further purification by free-flow electrophoresis, a single dansyl spot appeared at R_f 0.135 in solvent system 2° II, with no migration in the other solvent systems. The spot disappeared after acid hydrolysis and, as in the case of the BA peptide, it was replaced by monodansyllysine.

TABLE 6

AMINO ACID ANALYSIS OF BA PEPTIDE

Amino acid	pmoles/g	Equivalents
ala	16.25	1.18
gly	26.13	1.90
ile	13.75	1.00
leu	12.37	0.90
lys	30.25	2.20
phe	13.88	1.01
pro	12.37	0.90
ser	14.70	1.07
tyr	11.96	0.87
val	13.10	0.95

Pyroglutamic acid was not quantitated because only traces of it were converted to glu by acid hydrolysis. Equivalents were calculated on the assumption that the molecule contained a single ile.

Structural Analysis of the BA Peptide

Acid hydrolysis showed the presence of the following amino acids (besides pglu mentioned above); ala, gly, ile, leu, lys, phe, pro, ser, tyr, val. Digestion with AM did not reveal any acid-sensitive amino acid. Absence of phe and pro from the AM hydrolyzate suggested the presence of a phe-pro or pro-phe bond which could not be split by the enzyme. Theoretically, AM should not have acted on a peptide with blocked N-terminal but it did hydrolyze both the BA peptide and LRF which has pglu for N-terminus, probably owing to the presence of traces of other enzymes.

Quantitative amino acid analysis, by the method mentioned above, showed one residue for each of the amino acids, except for two gly and two lys (Table 6). This indicated a tridecapeptide with a molecular weight of 1389. Its concentration in the brain was estimated at 13.75 pmoles (19.0 ng) per g. The 720 g of brain equivalents available for analysis represented, therefore, 13.75 μg of pure peptide.

Samples of BA peptide were digested for varying intervals and at varying temperatures with 0.01 μg/μl of CPB. Digestion for 14 min at 28° C released lys and at 38° C lys and ser. After one hour's digestion, gly, ser, tyr and 2 lys were released and after 10 hr an additional leu was split off. The lack of phe indicated that it was N-terminal to pro. The partial sequence at this point appeared to be pglu-(ala,gly,ile,val)-phe-pro-leu-(gly,lys,tyr)-ser-lys.

Tryptic digestion did not reveal the amino linked with lys. Gray [10] has noted that the dansyl derivative of the N-terminal tyr is often difficult to detect and we have made similar observations on synthetic peptides. Chymotryptic digestion yielded fragments with N-terminal gly and traces of N-terminal lys. It is known that prolonged hydrolysis with chymotrypsin can split leu- bonds. This suggested the following partial sequence: pglu-(ala,gly,ile,val)-phe-pro-leu-lys-tyr-gly-ser-lys.

Although cathepsin C (CC) does not usually attack peptides with blocked N-terminal, it was found that prolonged digestion of LRF (which has pglu as N-terminal group) with high concentration of enzyme (2μg/μl for 72 h) yielded the expected dipeptides. Samples of BA peptides

TABLE 7

SUMMARY OF THE STEPS IN THE SEQUENTIAL ANALYSIS OF BA PEPTIDE

Procedures	Results
1. N-terminal analysis and AM digestion	pglu ———pro-phe or phe-pro ↓ ———
2. Mild digestion with CPB at 28°	pglu ———pro-phe or phe-pro ———↓lys
3. Mild digestion with CPB at 38°	pglu ———pro-phe or phe-pro ———↓ser ↓ lys
4. Moderate digestion conditions with CPB	pglu ———pro-phe or phe-pro ↓ (lys, tyr, gly) ↓ ser ↓ lys
5. Harsh digestion conditions with CPB	pglu ———phe-pro ↓leu ↓(lys, tyr, gly) ser-lys
6. N-terminal analysis of chymotropic fragments	pglu ———phe-pro- leu ↓ *lys*-tyr ↓ *gly*- ser-lys
7. N-terminal analysis of cathepsin C fragments	pglu—↓ *gly*———phe-pro- leu- lys-tyr- gly- ser-lys
8. N-terminal analysis of cathepsin C fragments (digested under harsh conditions)	pglu—↓ *gly* —↓ *val*-phe-pro- leu- lys-tyr- gly- ser-lys
9. Analysis of dipeptide fragment (italicized) in cathepsin C digest	pglu- ile-*gly-ala*-val- phe-pro- leu- lys-tyr- gly- ser-lys

Arrows indicate peptide bonds broken by the enzyme indicated. Italicized residues are N-terminals of enzyme digestion fragments.

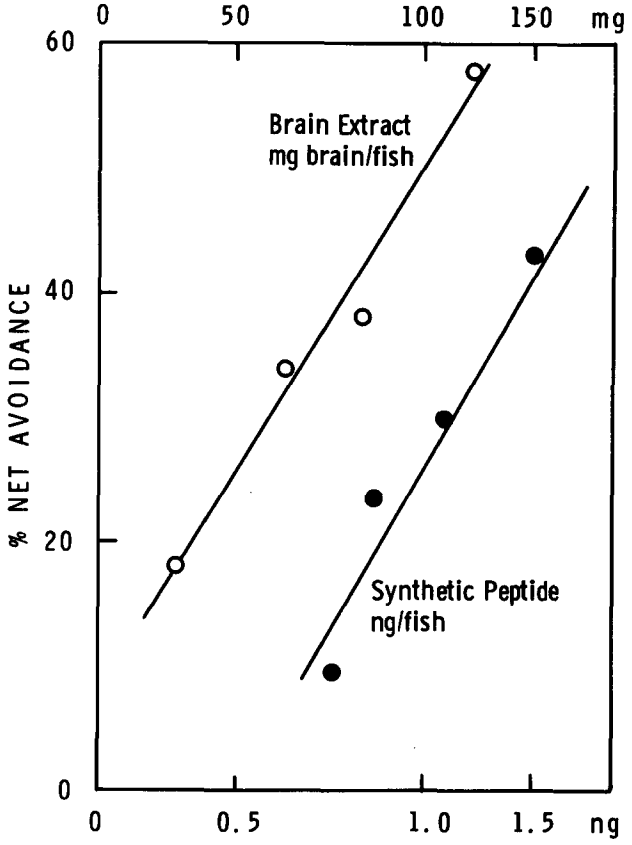

FIG.1. Dose-response curves of synthetic BA peptide and dialyzate of brain taken from blue avoidance trained fish. Abscissa, synthetic peptide in ng, brain equivalent of extract in mg (log scale). Ordinate, difference between mean net avoidance on Days 1 and 2 and mean net avoidance before injection (in percent). Each point represents the mean value for 8 fish, injected intracranially. Regression lines calculated by the least squares method.

were digested under the same conditions. The fragments released showed N-terminal gly followed by N-terminal val. Enzyme action was stopped at val because the next linkage, phe-pro, could not be split by CC. The N-terminal fragment of the BA peptide could therefore be either pglu-ala-gly-ile or pglu-ile-gly-ala.

To decide between the two alternatives, the dansyl derivatives of the dipeptides released by CC were compared with those of the commercially available synthetic dipeptides gly-ile and gly-ala. It was not possible to ascertain the presence or absence of gly-ala because its dansyl derivative is masked by the by-products of the dansyl reaction. However, DNS-gly-ile, which is easily detectable, was absent from several plates prepared from CC hydrolyzates. The following tentative sequence was therefore attributed to the BA peptide: pglu-ile-gly-ala-val-phe-pro-leu-lys-tyr-gly-ser-lys-OH. Successive steps of the sequential analysis of peptide BA are summarized in Table 7.

Analysis and Biological Assay of the Synthetic BA Peptide

The proposed sequence was synthesized by Dr. D. Sarantakis of Wyeth Laboratories. The synthesis was carried out by the solid phase method and the product was purified on the Sephadex G-25 column. The dansyl derivative of the purified synthetic peptide migrated on polyamide plates like the natural material. Quantitative analysis showed the same amino acids in the same proportions and enzymic breakdown yielded the same fragments. In all the respects that were tested, the synthetic product was chemically identical with the material isolated from the brain.

Figure 1 shows the results of bioassays carried out with the synthetic peptide under the conditions used for testing the crude extracts and the purification fractions. It shows that the doses increasing from 0.7 to 1.5 ng per fish induce dose-related blue avoidance behavior. Doses above the range shown in the figure produced lesser effects, as had been observed with other learning-induced peptides [4]. Comparison with the dose-response curve of the crude brain extract shows that 1 g of brain corresponds approximately to 20 ng of peptide.

Structural Analysis of the GA Peptide

Dansylation followed by acid hydrolysis showed that this peptide also had a blocked N-terminal. Mild alkaline

TABLE 8

AMINO ACID ANALYSIS OF GA PEPTIDE

Amino acid	pmoles/g	Equivalents*
ala	7.00	0.92
gln	5.90	0.77
gly	16.70	2.18
ile	7.64	1.00
leu	9.00	1.18
lys	13.70	1.79
phe	6.06	0.79
pro	6.96	0.91
ser	9.52	1.25
tyr	9.92	1.30
val	6.84	0.86

*Equivalents were calculated on the basis of ile = 1.0.

hydrolysis revealed didansyl-lysine, suggesting the presence of N-methyl-lysine or N-acetyl-lysine. The latter seemed the most probable because of the ease with which alkaline treatment removed the blocking group.

Qualitative and quantitative analysis after acid hydrolysis showed the same amino acid composition as that of the BA peptide (Table 8). Enzymic hydrolysis with AM showed, however, the presence of gln instead of glu. Assuming that the N-terminal group was NAc-lys, the molecular weight was estimated to be 1448.

The procedures used for sequential analysis of the GA peptide are summarized in Table 9. Digestion with CPB (0.01 μg/μl for 1 hr) released gly, lys, ser and tyr from the C-terminal of the molecule. Tryptic digestion for 12 hr yielded a fragment with N-terminal gly, indicating the presence of a lys-gly bond. Chymotryptic hydrolysis (0.05 μg/μl for 12 hr) released a fragment with N-terminal gly and traces of lys. With higher concentration of enzyme and more prolonged hydrolysis (0.2 μg/μl for 24 hr) an additional fragment appeared with N-terminal ile (under the same conditions chymotrypsin broke the gln-gln bond of substance P). This suggested the presence of leu-lys, gln-ile and either phe-gly or tyr-gly linkages. Since the second lys was known to be in the fragment C-terminal to pro, one could assume the presence of a pro-leu-lys sequence.

Existence of a phe-gly bond would suggest the following partial sequence: NAc-lys(gln-ile, phe-gly, ala, val) pro-leu-lys-gly-ser-tyr. In this case, however, tryptic digestion would have revealed a fragment with N-terminal gln, phe, ala or val. Failure to find such fragments and the fact that phe-pro is refractory to chymotrypsin and lys-tyr (as seen with the BA peptide) is undetectable by dansylation makes the following partial sequence more probable: NAc-lys-gly,gln-ile(ala,val) -phe-pro-leu-lys-tyr-gly-ser.

After unblocking the N-terminal with 1 N NaOH for 18 hr at 4° C, we submitted the peptide to CC (0.2 μg/μl for 24 hr). Fragments were released with N-terminal lys, gln, ala and traces of phe. The N-terminal fragment of the peptide could, therefore, be either NAc-lys-gly-ala-val-gln-ile-phe-pro or NAc-lys-gly-gln-ile-val-phe-pro.

To test these possibilities, a sample was digested first with trypsin (0.05 μg/μl for 12 hr) to release the fragment gly(gln-ile,val,ala)phe-pro-leu-lys. A further incubation with CC (0.2 μg/μl for 24 hr) gave dipeptides with the following N-terminals: gly, ile and val. (In the second incubation, trypsin was inactive because digestion was carried out at pH 5 and CC did not attack trypsin because it does not act on proteins [19]). The sequence of the tryptic fragment, therefore, was either gly-gln-ile-ala-val-phe-pro-leu-lys or gly-ala-val-gln-ile-phe-pro-leu-lys.

This still leaves two possible sequences for the whole peptide: Nac-lys-gly-gln-ile-ala-val-phe-pro-leu-lys-tyr-gly-ser-OH or Nac-lys-gly-ala-val-gln-ile-phe-pro-leu-lys-try-gly-ser-OH. Since our techniques cannot choose between the two alternatives, both sequences are currently being synthesized by Dr. H. Lackner of the University of Göttingen. Comparison of the synthetic products with the natural material should help to select the correct sequence.

DISCUSSION

The analytical methods used in the work reported in this paper were dictated by the extremely small amounts of material available for determining the structure of the peptides: less than 15 μg for the BA peptide and, only about 8 μg for the GA peptide. More conventional sequencing techniques would have required 20 to 50 times more material, involving the training of hundreds of thousands of fish for each of the two behaviors. The analytical methods based on the ultramicrodansylation, thin-layer chromatography and enzymic fragmentation initiated by Neuhoff *et al.* [20,21] and further developed in our laboratory by Burzynski [3,4] were, therefore, the only means by which the present work could be accomplished.

TABLE 9

STEPS IN THE SEQUENTIAL ANALYSIS OF GA PEPTIDE

Procedures	Results
1. N-terminal analysis and CPB digestion	N-Ac-lys ——————————pro ↓—(lys, tyr, ser, gly)
2. N-terminal analyses of chymotryptic and tryptic fragments	N-Ac-lys ↓ *gly*, gln ↓ *ile* (ala, val) phe-pro-leu ↓ *lys* ↓ *tyr* ↓ *gly*-ser
3. N-terminal analyses of cathepsin C fragments of unblocked peptide	lys-gly ↓ *gln*-ile ↓ *ala*-val-*phe*-pro-leu-lys-tyr-gly-ser lys-gly ↓ *ala*-val ↓ *gln*-ile-*phe*-pro-leu-lys-tyr-gly-ser (or)
4. N-terminal analysis of cathepsin C digest of a tryptic fragment	*gly*-gln ↓ *ile*-ala ↓ *val*-phe-pro-leu-lys *gly*-ala ↓ *val*-gln ↓ *ile*-phe-pro-leu-lys (or)
5. Alternative sequences	N-Ac-lys-gly-gln-ile-ala-val-phe-pro-leu-lys-tyr-gly-ser N-Ac-lys-gly-ala-val-gln-ile-phe-pro-leu-lys-tyr-gly-ser (or)

Arrows indicate the bonds split, italicized residues are N-terminals of fragments of enzymic hydrolysis.

The method has its limitations, as exemplified by the two equally possible sequences for the GA peptide. The limitations are partly those of the dansyl method, for example, the difficulty in detecting N-terminal tyr and the masking of DNS-gly-ala- by the by-products of the reaction) and partly due to the presence of peptide bonds that are resistant to enzymic attack (blocked terminals and pro linkages). In the present case, the N-terminal block could be overcome and the presence of pro in the middle of the molecule turned into an advantage by stopping the action of the enzymes at a convenient site. It is probable that further developments, such as availability of enzymes with highly selective bond specificities and use of more varied solvents systems for chromatography will extend the usefulness of the method. Its sensitivity has already been increased by several orders of magnitude by the use of isotope labeled dansyl reagents [3,4].

The area of research of the present study has been controversial from its beginnings, eleven years ago. The disputed points have been analyzed in several publications [7, 18, 28, 29]. In spite of the controversy, reports of successful bioassays, suggesting the formation of learning-induced substances in the brain, have been published to date from at least 42 laboratories in over 180 publications (see reviews [1, 7, 8, 12, 18, 24, 26, 32, 37]). Perhaps the most controversial aspect of the problem is the specificity of the brain material for the task learned, the so-called "one behavior, one peptide" principle [29]. Evidence has been produced for such specificity [16, 17, 22, 26] but much remains to be done to define its extent.

The present work indicates that the two opposite behaviors, blue avoidance coupled with green preference and green avoidance coupled with blue preference, are associated with the formation in the brain of two different but related peptides. They have a common sequence of 8 or 9 residues (according to which of the two proposed structures of the GA peptide is correct). The difference between them consists in the C-terminal lys of the BA peptide becoming N-terminal in the GA material and the 3 or 4 first amino acids switching sites. This suggests the possible existence of a whole family of peptides associated with color discrimination; they were given the collective name of chromodiopsins [33]. Future experiments, using different coupled colors, may give further insight into the rules of a possible coding system.

Hypotheses proposed for a molecular coding of neural information have been discussed elsewhere [25–27, 29]. Whatever the significance of the learning-induced peptides may be, they have considerable bearing on the future of behavioral pharmacology. Most of the present drugs act upon the affective background of behavior by modifying the balance of the synaptic transmitters or modulators. Owing to their practically unlimited information content, peptides could induce much more specific effects by modifying the cognitive content of behavior.

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