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The Effects of Dogfish MSH's and of Corticotrophin-like Intermediate Lobe Peptides (CLIP's) on Avoidance Behavior in Rats[1]

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Recently purified melanocyte-stimulating hormones (MSH's) from dogfish pituitary tissue were tested on extinction of a conditioned avoidance response (CAR). Corticotrophin like intermediate lobe peptides (CLIP's) from dogfish and porcine origin were tested for an effect on avoidance extinction as well. All peptides appeared to delay extinction of the CAR.

The results suggest that the pituitary contains various peptides which influence adaptive behavior. The observation that MSH is more potent in delaying extinction of the CAR then CLIP leads to the conclusion that the behavioral active sequence of the ACTH molecules is located in the N terminal part rather than in the C terminal part of the polypetide.

INTRODUCTION

It is well established that the pituitary gland plays an important role in adaptive behavior. Pituitary peptides affect acquisition and maintenance of conditioned behavior. Adenohypophysectomy (De Wied, 1964) or ablation of the whole pituitary (De Wied, 1968) markedly reduce the ability of rats to acquire a shuttle box avoidance response. Treatment with ACTH, or with α-MSH and fragments of the ACTH molecule (ACTH 1-10, ACTH 4-10) restores the rate of acquisition of the avoidance response towards nearly normal levels (De Wied, 1968; De Wied, van Delft, Gispen, Weijnen and van Wimersma Greidanus, 1972). ACTH, ACTH 1-10, ACTH 4-10, mammalian α-MSH and β-MSH also affect conditioned behavior in intact rats. These results suggest that the pituitary gland may contain a number of peptides which play a role in adaptive behavior (De Wied *et al.*, 1972; De Wied, 1974; De Wied, van Wimersma Greidanus and Bohus, 1974).

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Recently, several new or unusual peptides, related to ACTH, have been isolated from the pituitary gland. The purpose of this paper is to describe the effect of these peptides on avoidance behavior in rats.

Scott, Ratcliff, Rees, Landon, Bennett, Lowry, and McMartin, (1973) have demonstrated the presence of the 18-39 fragment of the ACTH molecule in the pars intermedia of the rat and pig pituitary. This peptide (Table 1), which is probably produced as a result of the formation of α-MSH from the same gene as ACTH, has been called the "Corticotrophin-like Intermediate Lobe peptide (CLIP)." Lowry, Bennet, McMartin and Scott (1974) have succeeded in demonstrating the presence of this same peptide in the pars intermedia of a dogfish (Squalus acanthias). Its amino acid sequence is substantially different from that of pig or rat CLIP (Table 1), although, of course, it is identical structurally to the 18-39 portion of the dogfish ACTH molecule. In the pars intermedia of the same animal, Lowry and Chadwick (1970) and Bennet, Lowry, McMartin, and Scott (1974) have demonstrated two unacetylated α-melanocyte-stimulating hormones which differ only by the presence or absence of a C terminal amide group. The same workers have also found a novel β-melanocyte-stimulating hormone (Table 1) which only contains the sequence of amino acids -His-Phe-Arg-Trp- in common with the 4-10 region of ACTH. The ACTH 4-10 sequence has been found in all the mammalian ACTH, MSH and Lipotrophin molecules that have been studied, and also in *Squalus acanthias* ACTH and α-MSH. Dogfish β-MSH nevertheless has an equivalent melanocyte-stimulating potency to the dogfish α-MSH's.

MATERIALS AND METHODS

Isolation and Identification of the Peptides

The isolation of the dogfish (*Squalus acanthias*) α-MSH and β-MSH, dogfish CLIP and porcine CLIP has been described elsewhere (Lowry and Chadwick, 1970; Lowry *et al.*, 1974; Scott, Lowry, Bennett, McMartin, and Ratcliffe, 1974). Extraction of the acetone dried neuro-intermediate lobes was performed by use of glacial acetic acid. The extracts were fractionated on a Bio-Gel P-2/P-6 system and MSH active peptides were submitted to ion-exchange chromatography on CM-cellulose for separation of the components. Repurification of dogfish β-MSH was achieved by ion-exchange chromatography on DEAE-cellulose. Dogfish CLIP was also purified on DEAE-cellulose.

Tryptic hydrolysis and pepsin digestion of the intermediate-lobe peptides were carried out, and the resulting peptide fragments were separated by ion-exchange chromatography on DEAE-cellulose and small amounts analysed for amino acid content in an auto-analyzer. Sequence analysis of the whole peptide was carried out by using the subtractive Edman degradation. For terminal analysis carboxypeptidases A and B and aminopeptidase M were used.

Behavioral Procedure

Male rats of an inbred Wistar strain weighing 110-130 gm were trained in a pole-jumping situation (van Wimersma Greidanus and De Wied, 1971). Briefly, the animals have to jump onto a pole, placed vertically in the middle of the box, in order to escape or avoid a scrambled foot shock (EFS) (0.25 mA) which is presented through the grid floor of the box. The conditioned stimulus (CS), involving the switching on of a 40 W light bulb fixed on top of the box, is presented 5 sec prior to the onset of the unconditioned stimulus (US) of EFS. As soon as the animal jumps onto the pole the CS and US are terminated. When the animal jumps in response to the CS alone, thus making a conditioned avoidance response (CAR), the light is switched off immediately and the US is not presented.

Each day, 10 trials are presented with a mean intertrial onset interval of 60 sec for three consecutive days. On the fourth day, extinction trials are run and the US is not applied anymore. The CS is presented maximally for 5 sec or terminated as soon as the animal makes the response. Animals which make at least 8 CAR's out of the 10 trials in the first extinction session are used for further experimentation. These animals are allocated to different treatment groups and sc injected immediately. Peptides are administered at different dose levels in 0.5 ml saline. A second and a third extinction session is performed at Day 4 at 2 and 4 hr after treatment. A fourth extinction session is run on Day 5, 24 hr after the single injection of peptide or placebo.

For statistical analysis the Kruskal-Wallis test and the *U*-test of Mann-Whitney are used (Siegel, 1965).

RESULTS

As can be seen in Tables 2 and 3, both dogfish MSH's display a strong dose dependent inhibitory effect on extinction of the pole jumping avoidance response at 2 and 4 hr after treatment. Amounts as low as 0.2 μgm induce a significant inhibition of extinction. The effect is more potent following treatment with doses of 1.0 μgm and still stronger after an injection of 5.0 μgm. Even at 24 hr after administration of the peptide, a small but significant inhibition of extinction can be observed following treatment with a 1.0 μgm or 5.0 μgm dose of dogfish β-MSH or even with 0.2 μgm dogfish α-MSH.

Porcine CLIP and dogfish CLIP appear to have behavioral effects as well, although these effects, in particular that of porcine CLIP, seem to be less potent than those of either MSH (Table 4). In order to compare the effects of CLIP with those of MSH, a group of animals treated with 1.0 μgm dogfish β-MSH was included in the CLIP experiments (Table 4).

TABLE 1

α-MSH	:	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
Dogfish α-MSH (= ACTH 1-13)	:	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Met-OH
Dogfish β-MSH	:	H-Asp-Gly-Asp-Asp-Tyr-Lys-Phe-Gly-His-Phe-Arg-Trp-Ser-Val-Pro-Leu-OH
Dogfish CLIP	:	H-Arg-Pro-Ile-Lys-Val-Tyr-Pro-Asn-Ser-Phe-Glu-Asp-Glu-Ser-Val-Glu-Asn-Met-Gly-Pro-Glu-Leu-OH
CLIP (porcine)	:	H-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH

TABLE 2

Effect of Dogfish α-MSH on Extinction of a Pole Jumping Avoidance Response

Treatment	Acquisition			Extinction				
	Day I	Day II	Day III	Day IV_1[b]	Day IV_2	Day IV_3	Day V	
5.0 μgm α-MSH	1.5±0.5[a]	6.1±0.3	8.6±0.2	8.8±0.2	9.0±0.3	6.4±0.4	4.1±0.3	(8)[c]
1.0 μgm α-MSH	1.0±0.3	5.1±0.5	8.1±0.3	8.9±0.2	7.4±0.3	4.6±0.4	3.3±0.5	(8)
0.2 μgm α-MSH	1.6±0.6	6.4±0.6	8.6±0.3	8.9±0.3	6.1±0.4	4.0±0.4	2.1±0.3	(7)
Placebo	1.0±0.2	5.7±0.4	8.0±0.2	8.7±0.2	4.3±0.2	2.4±0.2	1.0±0.3	(7)

[a]Mean number of CAR's ± SEM.
[b]sc injection.
[c]Numbers in parentheses are number of rats in treatment group.
*$p < .05$ (Mann Whitney's U test).
**$p < .001$ (Kruskal-Wallis' test).

TABLE 3

Effect of Dogfish β-MSH on Extinction of a Pole Jumping Avoidance Response

Treatment	Acquisition			Extinction				
	Day I	Day II	Day III	Day IV_1[b]	Day IV_2	Day IV_3	Day V	
5.0 μgm β-MSH	1.5±0.5[a]	6.3±0.4	8.0±0.2	8.3±0.2	8.5±0.8	7.0±0.6	5.0±0.6	(6)[c]
1.0 μgm β-MSH	1.5±0.3	7.3±0.4	8.7±0.2	8.7±0.2	7.7±0.5	5.2±0.4	3.2±0.5	(6)
0.2 μgm β-MSH	2.3±0.6	7.8±0.5	8.5±0.2	8.7±0.3	5.0±0.9	3.0±0.6	1.7±0.3	(6)
Placebo	1.4±0.5	6.4±0.3	7.7±0.2	8.5±0.2	2.4±0.7	1.2±0.3	0.7±0.2	(9)

[a]Mean number of CAR's ± SEM.
[b]sc injection.
[c]Numbers in parentheses are number of rats in treatment group.
*$p < .05$ (Mann Whitney's U test).
**$p < .001$ (Kruskal-Wallis' test).

TABLE 4

Effect of Porcine CLIP and of Dogfish CLIP on Extinction of a Pole Jumping Avoidance Response

Treatment	Acquisition			Extinction				
	Day I	Day II	Day III	Day IV_1[b]	Day IV_2	Day IV_3	Day V	
5.0 μgm Porcine CLIP	1.5±0.9[a]	5.8±1.4	8.5±0.3	8.5±0.5	7.2±0.2*	5.5±1.0*	3.8±1.0*	(4)
5.0 μgm dogfish CLIP	2.9±0.4	7.1±0.2	8.9±0.2	9.2±0.2	8.2±0.7*	7.1±0.7*	4.5±0.2*	(8)
1.0 μgm dogfish CLIP	3.4±0.9	7.8±0.4	9.2±0.4	9.4±0.2	6.6±0.5*	5.2±0.6*	3.4±0.2*	(5)
1.0 μgm dogfish β-MSH	3.7±0.9	7.2±0.5	8.5±0.3	9.5±0.3	7.5±0.9*	7.5±0.6*	5.8±1.1*	(4)
Placebo	1.0±0.3	6.5±0.6	8.2±0.3	9.0±0.2	5.0±0.4	2.9±0.5	0.4±0.2	(8)

[a]Mean number of CAR's ± SEM.
[b]sc injection.
*$p < .05$.

DISCUSSION

The melanocyte-stimulating hormones α- and β-MSH are probably best known for their effects on pigment cells in amphibians. MSH disperses melanin granules in amphibian melanophores. This darkens the skin of the animals, which can be regarded as an adaptive reaction. Among several extrapigmentary effects of MSH that have been observed in mammals are a number of behavioral effects such as: delay of extinction of a shuttlebox and of a pole jump avoidance response (De Wied, 1966; De Wied and Bohus, 1966; Greven and De Wied, 1967; 1973), prolonged extinction of a T-maze response for positive reinforcement (Sandman, Kastin, and Schally, 1969), facilitation of reversal learning performance (Sandman, Alexander, and Kastin, 1973), increased resistance to extinction of appetite tasks (Sandman *et al.*, 1969; Kastin, Dempsey, Le Blanc, Dyster-Aas, and Schally, 1974) and a delayed extinction of passive avoidance behavior (Sandman, Kastin, and Schally, 1971; Dempsey, Kastin, and Schally, 1972; Greven and De Wied, 1973). Other peptides from pituitary origin, besides ACTH and MSH have been reported to affect avoidance behavior as well. Vasopressin and desglycinamide-lysine vasopressin, (DG-LVP), a peptide which has been isolated from hog pituitaries (Lande, Witter, and De Wied, 1971) play an important role in avoidance behavior, particularly in memory processes (De Wied *et al.*, 1974; Van Wimersma Greidanus, Bohus and De Wied, 1975).

We have shown that dogfish α- and β-MSH have an inhibitory action on extinction of conditioned avoidance behavior which is a confirmation of earlier findings with synthetic α-MSH and with other ACTH analogues (De Wied, 1966; Greven and De Wied, 1967; 1973). The fact that dogfish β-MSH is equipotent with α-MSH on extinction of the avoidance response is of considerable interest. This MSH contains the tetrapeptide H-His-Phe-Arg-Trp-OH (ACTH 6-9). This peptide has been claimed as the smallest peptide from the N-terminal analogues of ACTH which exhibits the melanocyte-stimulating and the lipolytic activities (Otsuka and Inoye, 1964). However, when ACTH 6-10 (H-His-Phe-Arg-Trp-Gly-OH) alone was tested on avoidance behavior only a very weak effect was observed (Greven and De Wied, 1973). Structure activity relationship studies with ACTH analogues on avoidance behavior reveal that essential elements required for behavioral effects are not restricted to a single locus or active core, but are condensed in at least two regions of the molecule. It has been shown that one sequence which contains information essential for behavioral activity is the peptide ACTH 4-7: H-Met-Glu-His-Phe-OH (Greven and De Wied, 1973). However, the finding that ACTH analogues containing the amino acid sequence 7-9 influence extinction of avoidance behavior (De Wied *et al.*, 1975), indicates that the information for behavioral activity is not restricted to the locus ACTH 4-7, but is present in the sequence 7-9 as well. This latter amino acid sequence, which is also

present in dogfish MSH's (Table 1), seems to contain the behavioral activity in a dormant form which needs to be potentiated in order to show behavioral expression. This potentiation may be induced by lengthening of the peptide chain or by the introduction of modifications that are believed to make the molecule more resistant to metabolic degradation (De Wied *et al.*, 1975). It is also possible that the substitutions in positions 7, 8 and 13 in dogfish β-MSH (these correspond with positions 4, 5 and 10 in ACTH sequence) may have similar binding and biological properties at the behavioral receptors in the CNS. Indeed these substitutions were found to have little effect on the MSH activity of this peptide compared with dogfish α-MSH (Bennett *et al.*, 1974).

The finding that porcine CLIP and dogfish CLIP show inhibitory effects on avoidance extinction as well, supports our hypothesis that the pituitary contains various peptides which influence adaptive behavior by acting on the CNS. The fact that MSH is more potent in this respect than CLIP is in agreement with our suggestions that the behaviorally active sequence of the ACTH molecule is situated in the N terminal part rather than in the C terminal part of the polypeptide.

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