Dopamine acts on acetylation of proopiomelanocortin-derived products in dog pituitary

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Abstract. The effect of chronic dopaminergic receptor blockade using domperidone (DOM) on the immature dog pituitary content of POMC-related peptides was evaluated. Six immature dogs were treated with DOM for 15 days, 3 times/day, po (3 mg/kg) together with DOM sc (0.6 mg/kg) at 21.00 h. Placebo was administered to six control animals with the same protocol. On the 16th day, the animals were killed, the whole pituitary removed, homogenized, and submitted to reverse-phase HPLC purification prior to radioimmunoassay (RIA) evaluation of ß-endorphin, ACTH and α-MSH immunoreactivities (ir). DOM-treated dogs showed a pituitary concentration of ß-EP and ACTH similar to the placebo-treated dogs. The total α-MSH ir was similar in both groups and distributed on two main peaks: one corresponding to α-MSH and another coeluting with des-acetyl-α-MSH [1-13(ACTH)NH₃]. However, the percentage of α-MSH on total ir in DOM-treated dogs (15.4 ± 2.6%) was lower than in controls (37.5 ± 4.5%, P < 0.01); the corresponding percentage of 1-13(ACTH)NH₃ content was 63.0 ± 3.8% vs 44.7 ± 3.7%, (P < 0.01). The α-MSH/1-13(ACTH)NH₃ ratio was considerably decreased by the treatment (0.25 ± 0.06 vs 0.89 ± 0.15, P < 0.01). Acetyl ß-EP-like ir was also lower in treated (38.4 ± 5.4 fmol/mg) vs control (86.6 ± 19.2 fmol/mg, P < 0.05) animals. These data indicate that the dopaminergic system plays an important role in the control of acetylation processes of POMC-related peptides in the pituitary. This may be crucial as far as the biological activity of the peptides is concerned.

In a previous experiment we observed that two weeks of domperidone (DOM) treatment in sexually immature dogs stimulated the growth of adrenal zona reticularis and this was accompanied by the enhancement of delta-5 androgen response to ACTH (Perez-Fernandez et al. 1987). The mediators of this action are still to be defined, and hypothetically they could include prolactin, as well as other hormones which are under the tonic inhibitory control of dopamine, such as α-MSH (Tilders & Smelik 1977; Ben-Jonathan 1985).

α-MSH is part of a bigger precursor molecule, proopiomelanocortin (POMC), which contains within its structure ß-endorphin (ß-EP) (Eipper & Mains 1980) and other peptides, such as ACTH and pro-gamma-MSH (Lowry et al. 1983), which is able to promote adrenal trophism. Furthermore, α-MSH selectively stimulates zona glomerulosa growth (Robba et al. 1986) and adrenal androgens secretion from human adrenal cells in culture (Baird et al. 1983), although this is still a much debated question (Fujieda et al. 1981a). Moreover, in vivo studies indicate that the 3-fold increase in plasma ß-EP levels during prepubertal life, notwithstanding the steady ACTH concentrations, is significantly correlated to the increased delta-5 androgens secretion observed in the same children (Genazzani et al. 1983a,b).
In view of the above we have now repeated the DOM treatment in prepubertal dogs and examined pituitary changes of some POMC-related peptides.

**Materials and Methods**

**Protocol**

Twelve dogs, 6.1 ± 0.7 weeks of age (i.e. 5 before sexual maturation; 5 males and 7 females) (Schiebinger et al. 1981) from three different mothers were used. Six of them were treated with DOM po (3 mg/kg) 3 times a day (at 09.00, 14.00 and 21.00 h) together with DOM sc (0.6 mg/kg) at 21.00 h. The same protocol administering placebo, both po and sc, was applied to six genders. The treatment lasted 15 days. Twelve hours after the last DOM treatment, the animals were anaesthetized and killed by intracardiac formalin injection. The whole pituitaries were immediately removed, weighed, and boiled for 10 min in 0.05 mol/l acetic acid in order to block protease activities. The organs were homogenized in a Polytron and centrifuged at 12000 × g; the pellet was discarded and the clear supernatant dried and reconstituted with 0.4 ml acetonitrile/0.01 mol/l HCl (18:82). The possibility that artifacts were produced during the procedure was checked by boiling 10 µg of both α-MSH and 1-13(ACTH)NH₂ in acetic acid with or without a pituitary. Both peptides showed the same retention times after the different procedures. The quantitative analysis revealed that 95% of added peptides were recovered.

**High-performance liquid chromatography (HPLC).**

Homogenates (0.2 ml) were injected into an HPLC apparatus (Waters, MA) consisting of two pumps 510, a UV detector 440, and a gradient controller 680. A reverse-phase C-18 column (μBondapack, 3.9 × 300 mm, 10 micron size) was eluted in a linear gradient from 18% to 40% acetonitrile in 0.01 mol/l HCl, during 22 min, at a flow rate of 1.5 ml/min. The retention times of different peptides tested for at least 10 times over a period of six months were in min: 1-16EP = α-EP: 9.9 ± 0.4; 1-17EP = γ-EP: 13.5 ± 0.2; hβ-LPH (β-lipotropin): 15.8 ± 0.09; hβ-EP: 18.5 ± 0.1; acetyl-β-EP: 20.7 ± 0.2; 1-13(ACTH)NH₂: 8.1 ± 0.2; α-MSH: 10.4 ± 0.3; di-acetyl-α-MSH: 11.9 + 0.2; 1-39ACTH: 15.2 ± 0.2. Oxidized forms of both α-MSH and 1-13(ACTH)NH₂ show a retention time of 3.5 min.

For each sample, 48 fractions were collected (every 20 sec, starting from the 7th min of elution), dried and redissolved in 1 ml of 0.12 mol/l phosphate buffer, pH 7.4, and 0.1% bovine serum albumin.

In order to evaluate the amount of material sticking to the column, some peptides ng of each, were injected into the system and processed as above described. This experiment was repeated 5 times. Per cent recoveries were: 88.1 ± 10.4 for β-EP, 89.9 ± 12.4 for ACTH, 92.1 ± 11.4 for α-MSH, and 87.4 ± 8.1 for 1-13(ACTH)-NH₂.

![Graph](image)

**Fig. 1.**

Pituitary content of acetyl-β-EP, α-MSH and 1-13(ACTH)NH₂ in placebo- (open bars) and domperidone-treated (shaded bars) dogs. The α-MSH/1-13(ACTH)NH₂ ratio i also reported on the right. The values are expressed as mean ± standard error (*P < 0.01).
Radioimmunoassays

To test the hypothesis of possible artifacts during chromatographic procedures, a pituitary homogenate was subdivided into two equal aliquots and 5 mg of purified human B-lipotropin was added to one of them. The peptide was recovered by 81% and no apparent changes in β-EP were recorded.

Radioimmunoassays

Each fraction was tested for its β-EP-like immunoreactivity (ir) using the anti-β-EP serum B-4 (kindly donated by Dr V. Wiegant, Utrecht, NL) which cross-reacts with many β-EP-related peptides including acetyl-β-EP (100%) and hβ-LPH (110%). Thus, according to this β-EP ir spectrum and in view of the β-EP retention time, fractions were tested again using a specific anti-hβ-EP serum (kindly provided by Prof C. H. Li, San Francisco, CA) which does not recognize the above peptides, except for β-LPH (16.4%).

At the same time, all fractions were tested for their ACTH ir using antiserum and standard, both provided by the National Pituitary Program (NIH, Bethesda). The antiserum (West) cross-reacts at 100% with 1-39 ACTH, 6.7% with 1-24ACTH, 10.5% with 11-24 ACTH, and does not recognizes α-MSH, des-acetyl-α-MSH (1-13(ACTH)NH₂), and CLIP (18-39ACTH).

The first 17 fractions were also tested for their α-MSH ir, using standard and antiserum provided by Dr V. Wiegant. Anti-α-MSH serum (M2) cross-reacts 100% with α-MSH, and di-Ac-α-MSH 24.8% with desacetyl-α-MSH and less than 0.1% with other ACTH fragments. Details on β-EP (Genazzani et al. 1982) and ACTH (Facchinetti et al. 1983) RIA procedures have already been reported. α-MSH RIA was carried out using 100 μl M2 serum at 1/15 000 dilution, 25 μl of iodinated tracer (3000 cpm) and 50 μl of sample or standard. Incubation lasted 48 h at 4°C and free from antibody-bound tracer was separated through PEG 18%. Sensitivity was 2.5 fmol/tube. Intra-assay coefficient of variation was 7.0 ± 1.3%.

Quantitative values of individual peptides were calculated by adding the peak value of each one to those of the 2 adjacent fractions. If the ir peak of a given peptide was more than one min away of the expected elution time, its value were not taken into consideration.

Statistical analysis of the data were carried out using ANOVA and Wilcoxon tests.

Results

No differences were found in the pituitary weight of DOM-treated (14.8 ± 1.8 mg) and control dogs (16.3 ± 2.4 mg) (X ± SEM).

No changes occurred in the pituitary concentration of β-EP (14.1 ± 4.1 fmol/mg vs 11.1 ± 2.7), or
in 1-39ACTH content (34.8 ± 14.2 vs 31.4 ± 9.8 fmol/mg in placebo- and DOM-treated animals, respectively). In the mid-region of the chromatogram, close to the elution point of β-LPH, a poor immunoreactivity was observed. At the elution point of acetyl-β-EP (fractions 38–42), ir β-EP in treated dogs (38.4 ± 5.4 fmol/mg) was significantly lower than in placebo-treated dogs (86.6 ± 19.2 fmol/mg, P < 0.05) (Fig. 1).

α-MSH ir was mainly distributed on two peaks; one corresponding to α-MSH and the other one eluting at the same retention time of des-acetyl-α-MSH, i.e. 1-13(ACTH)NH₂. A smaller, but reliably present ir was observed close to elution time of di-Ac-α-MSH (Fig. 2).

Although the total α-MSH ir was similar in the two groups, the amount of des-acetyl-α-MSH was nearly doubled in the treated animals, whereas that of α-MSH was halved. This led to a significant (P < 0.01) reduction of the α-MSH/1-13(ACTH)NH₂ ratio in the DOM-treated group (Fig. 1).

Discussion

The dog pituitary, fractionated on HPLC and submitted to different RIA procedures, shows the presence of different POMC-related peptides, such as β-EP, ACTH, and α-MSH. These data, together with the presence of several compounds related to pro-enkephalin A and pro-enkephalin B in selected canine brain/pituitary regions show that, as in other mammals, the three opioid systems are present in the pituitary of the dog (Desiderio & Takeshita 1985). β-EP-like ir was distributed on the portion of the chromatogram where 1-31 β-EP and its smaller C-terminal fragments elute, whereas a poor reactivity, if any, was found in the β-LPH eluting region. A significant amount of ir β-EP (about 10% of the total) is present in correspondence to acetyl-β-EP retention time, but little ir was detected in the final portion of the chromatogram, where the C-terminal truncated forms of acetyl-β-EP elute. However, no valid conclusions could be obtained from these observations since the acetylated endorphins were only evaluated through a cross-reaction of anti β-EP serum (B4) and the elution programme of the HPLC was not especially designed to fractionate the different acetylated endorphin compounds.

Anyway, this pattern resembles that observed in human embryo (Facchinetti et al. 1987) and foetus (Vuolteenaho et al. 1983), but is different from that in the adult rat neurointermediate pituitary lobe (Smyth et al. 1979). α-MSH ir shows two major peaks, i.e. one corresponding to α-N-acetyl-α-MSH and the other one corresponding to des-acetyl-α-MSH, namely 1-13(ACTH)NH₂. Domperidone treatment induces specific changes in pituitary POMC-peptides. β-endorphin and 1-39ACTH contents remained unaffected in the treated group. On the other hand, although the total α-MSH ir remained unchanged, DOM-treated animals showed lower α-MSH and higher 1-13(ACTH)NH₂ values than controls. This leads to a reversal in the α-MSH/1-13(ACTH)NH₂ ratio which became very low in the treated dogs. Moreover, ir ascribed to acetyl-β-EP was significantly lower in treated vs control animals. It therefore seems that the blockade of peripheral dopaminergic receptors is accompanied by a decrease of α-N-acetyl POMC peptides.

It is well known that dopamine inhibits α-MSH secretion in the neurointermediate lobe (NIL) of different species (Bower et al. 1974; Leenders et al. 1986), and this action has been shown to be mediated by a D-2 dopamine receptor inhibition of POMC synthesis (Cote et al. 1986). Canine intermediate lobes probably behave in a similar manner, since the α-MSH content is decreased by the dopaminergic agonist and its plasma levels are stimulated by haloperidol (Kemppainen & Sartin 1986). However, two different cell types constitute dog-NIL (Halmi et al. 1981): one processes POMC like other mammalian NIL (producing α-MSH and β-endorphin), whereas the other one stains intensely for ACTH and only weakly for α-MSH. This differentiates dog-NIL from NIL in other species, except the rat NIL (Autelitano et al. 1985; Millington et al. 1986), and the ewe NIL (Smith et al. 1986) where the blockade of dopamine action increases α-N-acetylated POMC peptides. The data of the present study indicating that the dog pituitary has an opposite pattern in response to peripheral dopaminergic antagonism suggest the possibility that prepubertal dogs retain a pattern of processing similar to that which seems typical of foetal life in the mouse (Leenders et al. 1986) and in humans (unpublished data). On the other hand, the findings that domperidone
reduces both α-MSH and acetyl-β-EP pituitary content may be a consequence of an increased peptide release rather than a reduced acetylation activity. In fact, it has been shown in the rat that stress induces a selective release of acetyl-β-EP in respect to the other compounds (Akil et al. 1985).

The effect of dopamine on post-translational POMC processing can be physiologically significant, since acetylation greatly affects biological activity. The acetylated endorphins lose their opioid properties (Smyth et al. 1979), whereas the behavioural effects do not change.

In contrast, α-MSH has more potent behavioural (O'Donohue et al. 1982) and melanotrophic activities (Rudman et al. 1983) than its des-acetylated form. Moreover, there is the possibility that des-Acetyl-α-MSH could endogenously antagonize the effects of α-MSH (Mc Cormack et al. 1982).

The shift from α-MSH to des-acetyl-α-MSH observed after DOM treatment, is reminiscent of the situation observed in the human foetal pituitary, where des-Acetyl-α-MSH largely prevails over α-MSH (Tilders et al. 1981). It is worth noting that during foetal life, the adrenal cortex consists mainly of the zona reticularis, which produced delta-5 androgens instead of cortisol (Fujieda et al. 1981b). After birth, it involutes and slowly reappears years later only during adrenarche (Dohm 1973). We previously obtained morphological and functional maturation of this androgen secreting adrenal zone in immature dogs, through DOM treatment (Perez-Fernandez et al. 1987). It is therefore possible that the changes we now observed in the acetylation of POMC-peptides could play a role in this action.

In conclusion, these data demonstrated that the blockade of peripheral dopaminergic receptors interferes with the acetylation processes of POMC-related peptides and this could be of importance in the development of adrenal cortex in sexually immature dogs.

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