60 YEARS OF POMC

N-terminal POMC peptides and adrenal growth

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Abstract

The peptide hormones contained within the sequence of proopiomelanocortin (POMC) have diverse roles ranging from pigmentation to regulation of adrenal function to control of our appetite. It is generally acknowledged to be the archetypal hormone precursor, and as its biology has been unravelled, so too have many of the basic principles of hormone biosynthesis and processing. This short review focuses on one group of its peptide products, namely, those derived from the N-terminal of POMC and their role in the regulation of adrenal growth. From a historical and a personal perspective, it describes how their role in regulating proliferation of the adrenal cortex was identified and also highlights the key questions that remain to be answered.

Key Words

- ▶ N-POMC
- ▶ adrenal
- ▶ pro- γ -MSH
- ▶ growth
- ▶ POMC
- ▶ HPA axis

Journal of Molecular Endocrinology
(2016) **56**, T39–T48

Personal perspective

I first came across proopiomelanocortin (POMC) as a final year undergraduate student in a course called 'The impact of recombinant DNA technology on polyhormone precursors'. It was the early 1990s and the molecular revolution was in full swing. PCR was still a novelty, and it seemed that virtually everyday researchers were identifying new genes; as a naïve student, I had a keen ambition to become part of this. I think I learnt one big thing on that course and that was that technology drives science. I was fascinated by how as technology had advanced, from techniques such as the purification of peptides, pulse chase labelling, and cloning technologies, they had all been used to unravel the POMC story. Thus, when the chance came to undertake a PhD with Phil Lowry to work on this precursor molecule, it was not a difficult decision to embark on a career in the field of molecular endocrinology. My initial project aim was to investigate the potential differential processing of different variants of human POMC (Morris *et al.* 1995), but after about a year, the project shifted sideways into investigating the mechanisms by which POMC peptides regulate adrenal growth. I have worked in this area ever since.

Historical perspectives

Rather ironically, the early studies on the interactions between the pituitary and the adrenal were not concerned with steroidogenesis and the 'stress response'. Instead, they focused on a search for a factor that prevented adrenal atrophy. In the early part of the twentieth century, Ascoli and Legnani had shown that in dogs profound adrenal atrophy occurred following surgical removal (hypophysectomy) of the pituitary (Ascoli & Legnani 1912). In the early 1930s, Smith extended these studies by showing that the implantation of fresh pituitary tissue could reverse the same adrenal atrophy seen in hypophysectomised rats. He proposed that there must be a pituitary 'factor' that maintained the adrenal (Smith 1930).

Using adrenal weight as a bioassay, a number of researchers investigated the existence of a growth factor (Collip *et al.* 1933, Moon 1937, Simpson *et al.* 1943). However, the adrenal weight assay is incredibly insensitive requiring large amounts of pituitary extract to see an effect, and inevitably, this led to a quest to find a better bioassay. The first alternative was an assay developed by Sayers, which was based on the observation that levels of

ascorbic acid are depleted in adrenals following treatment with pituitary extracts (Sayers *et al.* 1944). Unknown at the time, it is actually ACTH that causes this effect (the significance of the depletion is still unclear), and with the further development in the 1950s of assays using corticosteroids as the endpoint, the scene was set to identify what we now call ACTH (Bell 1954, Howard *et al.* 1955, Shepherd *et al.* 1956).

With hindsight, it is probably fair to say that the change in bioassay, which resulted in the identification of ACTH, was a major factor in the loss of interest in the continued search for an adrenal growth factor. It was generally thought that there was no need, especially as early preparations purified from pituitary extracts that were capable of stimulating steroidogenesis were shown also to stimulate adrenal growth; of course, it was most likely that these preparations were contaminated with other (at that time unknown) POMC peptides. The lack of interest is evidenced by the fact that there is very little in the literature during the 1950s/1960s concerning an adrenal growth factor other than an interesting paper by Segal and Christy (Segal & Christy 1968). They exploited the fact that ACTH is quickly degraded in plasma and found that if they took plasma with high levels of ACTH (e.g. from patients with pituitary or ectopic tumors) and allowed it to stand at room temperature (to allow plasma proteolytic enzymes to exert their action), it was observed that although it could no longer stimulate corticosteroidogenesis, it could still maintain the adrenal weight in hypophysectomised rats. These results suggested that there was another factor, distinct from ACTH, which could stimulate adrenal growth.

Despite these early and now classical observations, throughout the 1970s and even more recently with the *Pomc* null mouse, opinion continued to very much favour ACTH as the single factor that stimulated adrenal growth. However, there are a number of other studies from this period that cast some doubt that this might not be entirely correct. The first of these was that ACTH quite clearly inhibited the growth of adrenal cells *in vitro* (Masui & Garren 1971, O'Hare & Munro-Neville 1973). The second was the observation that the treatment of rats with antiserum raised against ACTH, while decreasing blood corticosteroid levels, does not result in adrenal atrophy (Rao *et al.* 1978).

The year 1977 will be remembered as the landmark year in POMC research as it was the year that concurrently Mains *et al.* (1977) and Roberts and Herbert (1977) elegantly showed the existence of the precursor molecule and conclusively confirmed that ACTH and β -endorphin

were derived from the same molecule. These studies also showed that the precursor contained another, previously unknown glycopeptide that was named the '16K fragment' due to its apparent molecular weight when separated by SDS-PAGE. The name 16K fragment is rather erroneous, because the carbohydrate moiety significantly affects migration by SDS-PAGE and the molecular weight of the peptide is actually considerably less.

With the cloning of the gene encoding the precursor reported two years later (Nakanishi *et al.* 1979), the fine structure of the precursor was finally revealed. It was shown that all the peptides were flanked by the basic residues with ACTH located in the centre of the molecule, β -LPH at the C-terminal and the remaining residues, presumably the secretory signal sequence and the 16K fragment, located at the N-terminal of ACTH. The first purification of the 16K fragment from the media of AtT-20 cells (Keutmann *et al.* 1979) showed that the N-terminal residue of the mature peptide was tryptophan. This both explained the observation that pituitary tissue was fluorescent following treatment with formaldehyde (indicative of N-terminal tryptophan) (Håkanson *et al.* 1974) and subsequently formed the basis of an assay to allow the purification of the peptide from other species.

In humans, the 16K fragment is a 76-residue peptide with the sequence of γ_3 -MSH at its C-terminal (residues 50–76). This fact has resulted in the peptide often being called pro- γ -MSH (or N-POMC(1–76)). Several studies have shown that this peptide is one of the principal processed products secreted from the pituitary corticotroph cell into the circulation (Eipper & Mains 1978, Jackson *et al.* 1983).

The identification of N-POMC as a potential adrenal growth factor

Using tryptophan fluorescence as an assay, Estivariz and coworkers isolated human pro- γ -MSH from pituitary extracts (Estivariz *et al.* 1980). However, their initial experiments in which the peptide was administered either alone or in combination with ACTH to hypophysectomised rats showed it to be incapable of preventing adrenal atrophy. However, working under the hypothesis that other POMC peptides might be permissive for biological action, they subsequently showed that the treatment of intact rats with antiserum raised against pro- γ -MSH resulted in their adrenals having a decreased ability to incorporate ^3H thymidine: a marker of DNA synthesis and most probably cell division. In a later report (Estivariz *et al.* 1982), the authors treated isolated adrenal cells with two other peptides isolated from human pituitary extracts,

N-POMC(1–28) and N-POMC(2–59) (McLean *et al.* 1981), and showed that both resulted in a robust stimulation of DNA synthesis (as measured by thymidine incorporation) (Fig. 1). It is important to stress at this point that both of these peptides were thought to have been partial degradation products derived from pro- γ -MSH which were the result of proteolytic action in the mild alkaline conditions used in the large-scale extraction process for human growth hormone, from where the extracts had been derived. It was therefore felt that they were unlikely to represent any physiological relevant molecule (McLean *et al.* 1981).

Because the pituitary secretes pro- γ -MSH and the growth-promoting activity appears to reside in the N-terminal and only if the C-terminal was absent, a hypothesis was proposed that adrenal growth and mitogenesis were controlled by a postsecretional cleavage of pro- γ -MSH. It was suggested, quite logically, that the most likely cleavage site was at the arginine/lysine dibasic site at the N-terminal of γ_3 -MSH (Estivariz *et al.* 1982). Due to the incomplete understanding of prohormone processing at the time, it was often incorrectly assumed that cleavage at this site occurred after the lysine (reviewed by Harmer & Bicknell 2005), resulting in the formation of N-POMC(1–48) and γ_3 -MSH(51–76). In fact, cleavage occurs after the arginine and results in the generation of N-POMC(1–49) and Lys- γ_3 -MSH(50–76).

Figure 1

Stimulation of rat adrenal cells by various N-POMC peptides. Dispersed rat adrenal cells were incubated for 3 h before the addition of N-POMC(2–59), N-POMC(1–28), N-POMC(1–76) (pro- γ -MSH), or ACTH(1–24) at various concentrations. The cells were incubated for a further 3 h before the addition of 1 μ Ci of 3 H-thymidine. After a further 6 h, the medium was aspirated and 3 H-thymidine incorporation determined as a measure of DNA synthesis. Adapted by permission from Macmillan Publishers Ltd: *Nature* (Estivariz *et al.* 1982), copyright (1982).

Compensatory adrenal growth

The phenomenon of compensatory adrenal growth (Tepperman *et al.* 1943) has been used extensively to investigate the mechanisms underlying adrenal growth. The procedure in which one adrenal is surgically removed results in a rapid increase (within 24 h) in the size of the contralateral gland as measured by an increase in wet weight, and DNA and RNA synthesis. The mechanisms that regulate the process have been investigated extensively, and it has been demonstrated that a neural circuit exists involving the hypothalamus which when stimulated by manipulation of one adrenal stimulates growth of the other gland (Dallman *et al.* 1976, 1980). Despite the neural involvement, there is still a requirement for the pituitary because, although compensatory growth does occur in hypophysectomised animals, it is against the background of a gland undergoing rapid adrenal atrophy and appears more of a slowing of this atrophy rather than any increase in size (Dallman *et al.* 1980). It is perhaps not unreasonable to suggest that pro- γ -MSH would still be present in these experiments because, due to the disulphide bridge structure and glycan, it has a much longer half-life than the other POMC peptides such as ACTH.

To determine a definitive role of pro- γ -MSH in compensatory growth, Lowry and coworkers designed an elegant series of experiments in which antiserum was administered to different regions of POMC 2 h prior to unilateral adrenalectomy (Lowry *et al.* 1983). The remaining gland was removed 24 h postoperation, and the wet weight, DNA (as a marker of hyperplasia), and RNA (as a marker of hypertrophy) content compared. The results, summarised in Table 1, showed that an antibody against N-POMC(1–28) resulted in abolition of the increase in weight and DNA content, suggesting an inhibition in hyperplasia, whereas an antibody against ACTH resulted in a decrease in RNA content, suggesting an inhibition in hypertrophy. However, the most surprising result was seen when an antibody to synthetic γ_3 -MSH was used. This had the most dramatic effect, inhibiting the increase in weight and DNA and RNA content. Although pro- γ -MSH had been shown previously to cause an increase in adrenal RNA content (Al-Dujaili *et al.* 1982), the dramatic inhibition of the increase in DNA content during compensatory growth led to the proposal of a hypothesis by the authors that the antibody against γ_3 -MSH had resulted in the inhibition of the proteolytic cleavage needed to release the active growth factor. They also proposed that the enzyme that elicited this cleavage was likely to be under neural control.

Table 1 The effect of various POMC antisera on the compensatory growth response following unilateral adrenalectomy.

Antisera	Weight (%)	DNA (%)	RNA (%)	Corticosterone ($\mu\text{g}/100\text{ mL}$)
NRS	+32	+71	+83	33.3
ACTH	+36	+63	+10	15.7
N-POMC(1–28)	+21	0	+72	29.1
N-POMC(51–74) (γ_3 -MSH)	0	0	+19	16.0

Rats were injected with either normal rabbit serum (NRS) or antiserum raised against ACTH, N-POMC(1–28), or N-POMC(51–74) (γ_3 -MSH) 2 h before undergoing unilateral adrenalectomy. The animals were sacrificed 24 h later, and changes in wet weight, DNA content (as a marker of hyperplasia), and RNA content (as a marker of hypertrophy) were determined in the remaining gland compared to the excised gland. Plasma corticosterone levels were also determined and compared to levels before adrenalectomy. Data from [Lowry et al. \(1983\)](#).

Some experimental evidence supporting the hypothesis was obtained by injecting Trasylol (a trypsin-like enzyme inhibitor) into rats prior to unilateral adrenalectomy. Although the findings of these experiments have not been reported until much later ([Bicknell et al. 2001](#)), they showed that this inhibitor significantly decreased the increase in weight seen, supporting the hypothesis that a proteolytic cleavage was required during the growth response.

Other evidence for the role of N-POMC in adrenal growth

Besides compensatory growth following unilateral adrenalectomy, the adrenal cortex has the ability also to undergo regeneration following enucleation. In this model, the adrenal capsule is scored and the majority of the adrenal cortex, together with the medulla, is removed by squeezing the gland (so-called enucleation). The remaining tissue, consisting of the capsule and a few adherent cells derived from the zona glomerulosa, forms the precursor for complete regeneration of the cortex over a period of 4–6 weeks.

Estivariz and coworkers investigated the role for N-POMC in this model by removing the pituitaries of rats following bilateral enucleation and then administering either purified N-POMC(1–28) or ACTH by injection or continuous infusion using implanted minipumps ([Estivariz et al. 1988a](#)). The animals were then treated with colchicine before the adrenals were removed for the determination of mitotic counts on histological sections. The results showed that administration of N-POMC(1–28), but not ACTH, increased the mitotic index compared to saline controls, although ACTH, but not N-POMC(1–28), resulted in a significant increase in

corticosterone production. However, neither peptide resulted in an increase in the wet weight of the adrenals, suggesting that neither N-POMC(1–28) nor ACTH was, on their own, capable of rescuing the adrenal in the absence of all the POMC-derived peptides.

In the same study, the authors also compared the actions of purified N-POMC(1–28), synthetic N-POMC(1–28), and synthetic N-POMC(1–36) on thymidine incorporation in dispersed rat adrenal cells and found that the synthetic peptides were significantly less active than the purified N-POMC(1–28). They also tested the effects of the peptides *in vivo* by administering them to intact rats *via* osmotic minipumps. Examination of histological sections showed, compared to saline controls, an increase in mitotic counts in the animals treated with the purified peptide with a smaller effect with synthetic N-POMC(1–28). By contrast, N-POMC(1–36) had no significant effect at all. Because it would be expected that the three peptides would have had similar activity, these results were unexpected and the authors proposed that the inactivity of the N-POMC(1–36) was most likely to have been the result of the peptide having the disulfide bridge arrangements in the 2–8 and 20–24 configuration rather than the natural 2–24 and 8–20 arrangement ([Bennett 1984](#)).

In the same edition of *Journal of Endocrinology*, the same group ([Estivariz et al. 1988b](#)) reported a follow-up study in which they tested the effect of antiserum against either N-POMC or ACTH on mitotic activity in intact rats undergoing adrenal regeneration following enucleation. In these experiments, they found that only the N-POMC antibody decreased significantly mitotic counts, whereas the ACTH antibody resulted only in decreased corticosterone production. Interestingly, the authors also showed gel filtration data to suggest that the processing of POMC in the anterior pituitary changed during regeneration from predominantly pro- γ -MSH to a shorter form that eluted in a similar position to synthetic N-POMC(1–48). This result would therefore argue against the hypothesis of the need for a postsecretional cleavage of pro- γ -MSH, at least during regeneration.

There is some further evidence from sheep to support the notion that the processing of pro- γ -MSH can change ([Saphier et al. 1993](#)). In the sheep, parturition is initiated by a rapid rise in the levels of fetal plasma cortisol, and measurement of fetal ACTH levels showed them to be low during gestation and to increase moderately toward the end of gestation. By comparison, levels of pro- γ -MSH were shown to be relatively much

higher, but these dramatically decreased toward the end of gestation with a concomitant rise in the level of γ -MSH, suggesting that cleavage of pro- γ -MSH was occurring. However, whether the reduction in the levels of pro- γ -MSH was the result in changes in pituitary (or placental) processing was not determined.

Identification of an enzyme that cleaves pro- γ -MSH during compensatory growth

I first became interested in adrenal growth when, as part of my PhD project, I used differential display PCR (Liang & Pardee 1992) to generate RNA expression profiles from normal adrenals, glands undergoing compensatory growth, and also adrenals following bilateral enucleation. The ultimate aim of the project was to identify candidates for the enzyme that cleaves pro- γ -MSH, but unfortunately the approach was unsuccessful. The opportunity arose to continue the project as a postdoctoral fellow, and this time a more targeted approach was taken by using degenerate oligonucleotides designed around the conserved aspartic acid and serine residues that make up the catalytic triad of the trypsin family of serine proteases. Using these together with cDNA derived from adrenals undergoing compensatory growth, we identified a transcript that was upregulated during compensatory growth (Fig. 2). Cloning and sequencing of the full-length gene revealed it to be, at the time, an unknown member of the trypsin family of enzymes. The enzyme had a putative secretory signal sequence at the N-terminal, and because of this, we named it 'adrenal secretory protein' (abbreviated AsP).

We then conducted a number of experiments to investigate if AsP was involved with adrenal growth. Initially, we found that it was expressed in the outer adrenal cortex, mainly in the capsule and zona glomerulosa: the region where cellular proliferation occurs. We then carried out a number of experiments in mouse Y1 adrenal cells after we showed that the growth of these cells was, in part, dependent on the protease activity because their rate of growth was significantly decreased by treating them with the protease inhibitor, Trasylol. We subsequently showed that if the expression of the mouse equivalent of AsP was decreased using antisense RNA, then the rate of growth of the cells could also be significantly reduced, especially when the cells were grown in a minimal medium.

Although these experiments showed a role for AsP in growth, it was unclear if the enzyme could cleave pro- γ -MSH. All the evidence had suggested that the likely cleavage site was the dibasic RK site at the N-terminal of

Figure 2

Identification of trypsin-like serine proteases expressed in the rat adrenal following unilateral adrenalectomy. Rats underwent either unilateral adrenalectomy or a sham operation and were sacrificed either 6 or 24 h postoperation. The RNA from the remaining gland was extracted and used to synthesise cDNA that was subsequently used as a template in the PCR with degenerate oligonucleotides designed around the conserved residues of the active site of the trypsin family of enzymes. PCR products were labelled with ^{33}P during synthesis, separated by polyacrylamide gel electrophoresis and subsequently cloned and sequenced. Band 1 was identified as tissue plasminogen activator, band 2 as an unknown sequence that we named AsP, and band 3 as adipsin. Reprinted from *Cell*, Vol 105, Bicknell AB, Lomthaisong K, Woods RJ, Hutchinson EG, Bennett HP, Gladwell RT & Lowry PJ, Characterization of a serine protease that cleaves progamma-melanotropin at the adrenal to stimulate growth, Pages 903–912, Copyright (2001), with permission from Elsevier.

γ -MSH (Estivariz *et al.* 1982), so after affinity purifying AsP from the conditioned cell media, we tested this hypothesis using a quenched fluorescent substrate designed to represent the sequence around this cleavage point. Analysis of the cleavage products by mass spectrometry, much to our surprise, showed that cleavage appeared to be downstream between valine 52 and methionine 53, suggesting the active fragment to be N-POMC(1–52) (Bicknell *et al.* 2001).

At around the time this work was published, a human gene similar to AsP appeared on the database. This had been isolated from the sputum of patients with chronic airway disease and was named human airway trypsin-like protease (HAT). It had an identical catalytic subunit to AsP but instead of having a secretory signal sequence, contained a type II transmembrane domain at the N-terminal (Yasuoka *et al.* 1997). The question of whether HAT represented the human homolog of AsP was addressed by Hansen *et al.* (2004). They showed that although the rat adrenal predominantly expressed the shorter AsP, the mouse and human adrenal mainly expressed the longer, membrane-bound form. They also showed that beyond expression in the adrenal, there was extensive expression in the upper gastrointestinal tract and airways. This study showed clearly that there are distinct species differences and that this family of proteases has a multifunctional role beyond just adrenal proliferation.

Signaling and intracellular actions of N-POMC peptides in the adrenal

A number of groups have investigated the signaling events initiated by N-POMC in various adrenal systems. The first reported study (Fassnacht *et al.* 2003) showed that synthetic N-POMC(1–28) with the correct native disulphide bridge arrangement (Bennett 1984) could stimulate, in a dose dependent manner, proliferation of primary bovine adrenal cells, Y1 cells, and the human NCI-H295-R adrenal cell line. The authors also showed that the peptide could increase the phosphorylation of extracellular signal-regulated kinases (ERKs) 1 and 2, but there was no evidence of activation of the c-Jun or p38 pathways. They also showed that in H295R cells, the peptide decreased steroid hormone production.

We carried out a similar study (Pepper & Bicknell 2009) concentrating on Y1 cells and showed stimulation by both synthetic N-POMC(1–28) and purified N-POMC(1–49) resulted in an increase in phosphorylation of both ERKs 1 and 2 as well as their upstream regulators, MEK and c-RAF.

An *in vivo* study (Torres *et al.* 2010) showed that both N-POMC(1–28) and a modified version of the peptide with disrupted disulphide bridges could promote bromodeoxyuridine (BrdU) incorporation in both hypophysectomised and dexamethasone-treated rats and that the peptides protected against apoptosis. Further studies by the same group (Mattos *et al.* 2011, Mendonça & Lotfi 2011, Mendonça *et al.* 2013) subsequently showed that administration of N-POMC(1–28) stimulated proliferation *in vitro* of isolated rat adrenal cells *via* activation of the ERK pathway and *in vivo* upregulated the expression of cyclins D1, D2 and E, molecules that are all key mediators in the initiation of the cell cycle.

These studies demonstrate the ability of N-POMC (1–28) to stimulate intracellular signaling pathways, upregulate molecules involved with cellular proliferation, and induce cell growth. However, one criticism is that due to the relative ease of synthesis, all these studies have used N-POMC(1–28) rather than any of the longer fragments. It is quite possible that similar experiments utilising the longer N-terminal fragments might show them to be more potent and have larger biological effects.

The identity of the biologically active fragment of pro- γ -MSH that acts as an adrenal growth factor

As I have already described, data from both *in vivo* and *in vitro* had shown that pro- γ -MSH was (in terms of growth)

biologically inactive and that the active fragment is located in the N-terminal of the molecule. It was always thought likely that the most likely processing site for generation of such a fragment was the dibasic RK site at the N-terminal of γ -MSH (Estivariz *et al.* 1982). When we identified AsP, we worked under this hypothesis and tested the ability of the enzyme to process a small fluorescent peptide substrate, the sequence of which was derived from the sequence around this cleavage site. In retrospect, we should have digested the full-length pro- γ -MSH, but the presence of the glycosylation would have made it difficult to characterise the digestion products by mass spectrometry. As a result, the question has remained as to whether there was potentially any other processing sites in the molecule and whether the structure of the full-length molecule has any influence on the site of cleavage.

It is thus quite possible, and I think likely, that the adrenal growth factor might actually be a longer fragment. In the study by Estivariz and coworkers, they showed that the most potent stimulator of thymidine incorporation was N-POMC(2–59) (Estivariz *et al.* 1982). This peptide therefore contained the majority of the sequence of γ_1 -MSH, suggesting that its presence does not destroy the growth-promoting activity. It is interesting to note that the sequence of POMC up to C-terminal of γ_1 -MSH is highly conserved, but the sequence beyond this (i.e. the remaining dozen or so residues of pro- γ -MSH), apart from the N-linked glycosylation consensus sequence, shows considerable species variation, suggesting that there is evolutionary pressure on the upstream N-terminal fragment.

Taken together, this evidence might therefore suggest that the cleavage point of the molecule is actually at the C-terminal of γ_1 -MSH. If this is correct, it does pose the interesting question of how the remaining 11 residues or so of the C-terminal of pro- γ -MSH block the growth promoting activity? It might be a direct result of the sequence preventing receptor binding, but because this sequence also contains the bulky N-linked glycan, I would suggest that it is more likely that it is the presence of the glycan that prevents receptor binding/activation.

The *Pomc*^{-/-} mouse

With the perfection of the technology to specifically disrupt the function of a gene, it was no surprise when the first report came in 1999 (Yaswen *et al.* 1999) of a *Pomc* null mouse. This model, based on the 129 genetic background, had exon 3 of the *Pomc* gene disrupted, resulting in the loss of all but the first 18 residues of the mature protein.

A second group (Smart & Low 2003) generated a similar mouse based on the C57BL/6 background and a third group (Challis *et al.* 2004) independently generated a further *Pomc* null mouse on the 129 genetic background. In this last model, the authors not only deleted exon 3 but, in addition, also mutated the initiation codon. As a result, these animals would not be expected to produce any part of the POMC protein.

In all three models, homozygous mutant mice were born at a much lower frequency than expected, suggesting that disruption of the gene, perhaps unsurprisingly, is not compatible with life in most of the animals. However, whether this is due to a lack of POMC peptides or adrenal steroid hormones is unclear (Smart & Low 2003). Those animals that were born became obese with a number of other metabolic disturbances and, on the 129 genetic background, had also a yellow tinge to their fur. On both backgrounds, animals were found to have an absence of (or severely atrophied) adrenal tissue and no measurable corticosterone or aldosterone. The fact that these animals can survive at all without these steroids is unusual, as this scenario results in death in most other animals.

The *Pomc* null mouse, in a similar way to hypophysectomised animals, could potentially provide an ideal model in which to perform reconstitution studies and therefore investigate the role of different POMC peptides in adrenal function. The first such reported study (Smart & Low 2003) gave twice daily injections of 1 µg of ACTH(1–24) to *Pomc* null mice but found that this treatment did not result in the restoration of adrenal function. To put this into perspective, the basal circulating levels of plasma ACTH in mice are in the 100 pg/mL range (Muglia *et al.* 2000, Laurent *et al.* 2002). A dose of 1 µg could potentially achieve a theoretical maximum concentration in the animal of approximately 30 ng/mL (based on a 30 g body weight) or around 300 times basal levels. It would therefore seem reasonable that the lack of the activity of ACTH even at this relatively high dose would suggest that other POMC peptides beyond ACTH are required for normal adrenal function.

In a subsequent study (Coll *et al.* 2004), *Pomc* null mice were injected on a daily basis for a period of 10 days with an even higher dose of 30 µg of ACTH(1–24) (this dose would equate to a theoretical maximum concentration of approximately 1 µg/mL or around 10,000 times basal levels). The treatment did result in an increase in adrenal size and also restoration of corticosterone but not aldosterone levels. However, examination of the adrenals showed that the increase in size was a result of cellular hypertrophy with no evidence of any hyperplasia.

A carefully conducted study by Ute Hochgeschwender's group (Karpac *et al.* 2005) showed that the adrenals in the *Pomc* null mice developed normally but subsequently underwent atrophy after birth due to a lack of cellular proliferation rather than apoptosis. The authors also attempted to acutely stimulate the adrenals in young *Pomc* null mice with 1 µg of ACTH and found that although the adrenals at this stage appeared relatively normal, expressing both the ACTH receptor and components of the steroidogenic pathway, they found (as in the earlier study by Smart and Low) that the adrenals were not capable of secreting any corticosterone.

However, perhaps the most significant finding reported in this study was an experiment in which the authors transplanted single adrenals from 9-day-old *Pomc* null mice into bilateral adrenalectomised wild-type littermates, thereby creating a scenario in which the mutant adrenals were exposed to physiological levels of all of the POMC peptides. After a period of 3 months, it was found that the transplanted adrenal appeared to be histologically normal and fully functional producing normal levels of both aldosterone and corticosterone. This result clearly demonstrates the important fact that the mutant adrenals have the potential to become fully functional glands when exposed to an environment providing all (i.e. not just ACTH) the POMC peptides.

With the most obvious POMC peptide likely to be required to restore adrenal function being derived from the N-terminal of POMC, Coll and coworkers (Coll *et al.* 2006) carried out a further reconstitution experiment in which they injected *Pomc* null mice for a period of 10 days with 10 µg of synthetic N-POMC(1–28) (twice daily) or 30 µg of ACTH(1–24) (daily) either alone or in combination. In an identical manner to the earlier study (Coll *et al.* 2004), the pharmacological dose of ACTH resulted in significant adrenal cellular hypertrophy with no cell proliferation as assessed by proliferating cell nuclear antigen (PCNA) protein levels. By contrast, treatment with N-POMC(1–28) alone had no apparent effect on the adrenals with no increase in size or changes in PCNA expression. When both peptides were administered together, the adrenals were identical to when treated with ACTH alone.

The results of these experiments could be viewed as evidence against a role for the N-terminal peptides in adrenal growth, but, in my opinion, they should be interpreted with some caution.

In the study described earlier (Estivariz *et al.* 1988a), it was shown that in enucleated rats that had undergone hypophysectomy, treatment with N-POMC(1–28), although stimulating an increase in mitotic index, was

not capable of stimulating any increase in adrenal weight. Because N-POMC(1–28) is thought to be an extraction artefact and all the evidence, suggesting that the source of the active N-terminal fragment is pro- γ -MSH, it would seem that the choice of N-POMC(1–28) might have been erroneous. It could be reasonably suggested that treatment with pro- γ -MSH would have been a better choice.

Second, the doses of peptides used in the Coll study were in the pharmacological range and, as has already been mentioned, more physiological levels of ACTH do not restore adrenal function. It would thus not be inconceivable that the large doses of ACTH mask a more subtle effect if the two peptides were administered together in more physiological amounts.

A final point is that although the N-POMC(1–28) used in the study was synthesised with the correct disulfide bridge arrangements and had been shown previously to be biologically active (Fassnacht *et al.* 2003), the disulfide bridge arrangements in N-POMC are known to be unstable (Lowry 2015). It is thus possible that a rearrangement of the bridges to the 2–8, 20–24 configuration could have occurred and rendered the peptide biologically inactive as found in N-POMC(1–36) (Estivariz *et al.* 1988a).

The N-POMC receptor

It is beyond reasonable doubt that N-POMC plays a role in adrenal physiology, the data from the experiments I have described before have shown a role *in vivo*, and experiments both *in vivo* and *in vitro* have shown that the shorter peptides activate specific signaling pathways. One of the big questions remaining unsolved is the identity of the actual cell-surface receptor that N-POMC activates.

I made my first attempt to identify the receptor in 2002. Using N-POMC(1–28) attached to a solid phase support, I attempted to affinity purify the receptor from cell membranes derived from Y1 cells that had been solubilised with digitonin in a similar manner described by Gibbins and coworkers (Gibbins *et al.* 1996). On the first attempt, elution of the affinity column and separation of the proteins by SDS-PAGE revealed the presence of two bands of approximately 60 and 30 kDa, but we were unsuccessful in obtaining sequence data. However, I did manage to obtain some sequence from a subsequent experiment (Bicknell 2002), and although I thought initially it was a likely candidate, it eventually transpired that I had identified a protein involved in protein trafficking. After several more failed attempts to identify a candidate, it was decided to abandon this approach.

We made one further attempt to identify a receptor using degenerate primers and the PCR with cDNA derived from both Y1 cells and whole rat adrenal. However, once again no likely candidates were identified (Pepper & Bicknell 2009).

This was how the situation remained until last year, when I was lucky to be joined by Pedro de Mendonca, a postdoc from Claudimara Lotfi's lab in São Paulo, Brazil. I had first met Pedro at the 2012 Adrenal Cortex meeting, and he had expressed a keen interest to renew efforts to identify the receptor. After securing funding, he came to the UK on a 1-year fellowship to pursue this goal.

Using a microarray dataset kindly provided by Peter King (William Harvey Research Institute, Queen Mary University of London), together with data in the literature we compiled a list of orphan G-protein-coupled receptors expressed in the outer regions of the adrenal cortex. By overexpressing the receptors in HEK293 cells and then using a combination of molecular panning and ligand binding assays, we systematically screened them for the ability to bind to and be activated by N-POMC(1–28). Using this approach, we identified one receptor that gave a positive result in all of the assays. With a very strong candidate for the receptor identified, we are now continuing to fully characterise it in terms of its ligand specificity and investigate its role in adrenal physiology.

Conclusion

It is now over 100 years since it was first realised that the pituitary plays a role in the regulation of adrenal growth. The early attempts to identify this pituitary factor clearly illustrates that the choice of assay was (and still is) a critical decision. Ultimately, you only find what you look for and the quest to find a more efficient bioassay inadvertently led researchers to discover a different hormone—ACTH.

The elucidation of the structure of POMC in the late 1970s and the discovery that the precursor contained other peptides that had not been previously characterized opened several new avenues of research. One of these new avenues has been to elucidate the role of the N-terminal 16K fragment or pro- γ -MSH. The evidence overwhelmingly points to a role for this fragment in the regulation of adrenal growth, but even though it is over 30 years since the initial observations, the exact way that this is achieved is far from clear. It is still not fully understood how its activity is regulated, and up until very recently, there has been no convincing candidate for a receptor for it to work through. There is little doubt that a full characterisation

of this receptor will provide further clues into the role of N-POMC and adrenal growth and perhaps allow us to finally unravel the biology of this fascinating peptide.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

The writing of this review did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

Acknowledgements

I acknowledge the contributions of the individuals who have contributed to my work on N-POMC, especially Khomsorn Lomthaisong, Steve Harmer, David Pepper, Pedro de Mendonça, and Phil Lowry.

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Received in final form 6 January 2016

Accepted 12 January 2016

Accepted Preprint published online 12 January 2016