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Melanotrope Secretory Cycle is Regulated by Physiological Inputs via the Hypothalamus

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Running head: Regulation of melanotrope cell secretory cycle.

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Previously, it has been shown that background color conditions regulate the overall activity of the frog intermediate lobe by varying the proportions of the two subtypes of melanotropes existing in the gland, the highly active or *secretory* melanotropes and *hormone-storage* melanotropes, depending on melanocyte-stimulating hormone (α-MSH) requirements. However, the factors and mechanisms underlying these background-induced changes are still unknown. In the present study, we investigated whether hypothalamic factors known to regulate melanotrope cell function can induce changes *in vitro* similar to those caused by background adaptation *in vivo*. We found that the inhibitors apomorphine (a dopamine receptor agonist) and NPY decreased the number of active melanotropes and increased simultaneously that of storage melanotropes. On the other hand, the stimulator TRH increased the number of active cells and concomitantly reduced that of storage cells. Inasmuch as none of these treatments modified the apoptotic and proliferation rates in melanotrope cells, it appears that these hypothalamic factors caused actual interconversions of cells from a subpopulation to its counterpart. When taken together, these findings suggest that the hypothalamus would control melanotrope activity not only through short-term regulation of hormone synthesis and release, but also through a long-term regulation of the secretory phenotype of these cells whereby the activity of the intermediate lobe would be adjusted to fulfill the hormonal requirements imposed by background conditions.

Keywords: dopamine, melanotrope cell heterogeneity, NPY, secretory cycle, TRH.

IN AMPHIBIANS, melanotrope cells of the intermediate lobe regulate a unique physiological process, the adaptation of skin pigmentation to background color conditions (1, 2). Thus, high plasma levels of the hormone produced by these cells, melanocyte-stimulating hormone (α-MSH), induces the dispersion of the pigment melanin in dermal melanophores causing darkening of the skin, whereas low level of α-MSH prompts aggregation of the pigment and, consequently, skin paling. In a series of studies carried out in our laboratory, we have demonstrated that in the frog *Rana ridibunda* the overall activity of the intermediate lobe is adjusted by the proportions of two melanotrope cell subtypes, named as high- (HD) and low-density (LD) melanotropes based in their sedimentation characteristics in Percoll density gradients (3-5). According to their morphofunctional characteristics, HD melanotropes can be defined as a *hormone-storage* cell subset, because they show high α-MSH intracellular content but low mRNA levels of the prohormone precursor proopiomelanocortin (POMC) as well as a low basal α-MSH secretory rate, whereas LD cells show opposite features, indicative of high biosynthetic and secretory activities, and can therefore be considered as *secretory* melanotropes. Furthermore, we have also shown that the secretory LD melanotrope subpopulation largely predominated over the hormone-storage HD subset in the intermediate lobe of black background-adapted frogs (6, 7) which require high plasma α-MSH levels to sustain skin darkening. In contrast, the proportions of the two cell subsets were balanced under conditions of lower hormonal demand, in white-adapted animals (6, 7). These changes in the proportions of *secretory* and *storage* melanotropes between the two distinct physiological conditions occurred in the absence of variations in the apoptotic

and proliferation rates of cells, which suggested that melanotropes from a subpopulation convert into their counterparts according to the color of the background (6, 7). In other words, that melanotropes can pass from a relatively inactive (i.e. HD cells) to an active cellular state (i.e. LD cells) in response to the physiological demands of α-MSH.

These results have led our group to propose that, as suggested for other endocrine cell types (8-14), the morphologically and functionally distinct subtypes or subpopulations of cells that comprise the total population of melanotropes are in fact the reflection of different phases that occur at a given point in time within a dynamic and complex secretory cycle, which would operate, albeit with diverse specific features, in any endocrine cell type. Nevertheless, the mechanisms that govern the acquisition of a particular state of cellular activity and therefore determine the conversion of cells from a subpopulation to the other remain to be elucidated. Inasmuch as in amphibians melanotrope cell activity is directly regulated by the hypothalamus (for review see 15), it is conceivable that hypothalamic neurohormones modulate the timing of this secretory cycle. Accordingly, the aim of the present study has been to ascertain whether known hypothalamic regulators of the frog intermediate lobe, such as the inhibitors dopamine (15-18) and NPY (15, 17-23), and the stimulator TRH (15, 24-26), are able to induce the interconversion of cells from the two frog melanotrope subpopulations, as well as to analyze the relationship between the control of the dynamics of the melanotrope secretory cycle and background color adaptation.

MATERIALS AND METHODS

Reagents

Leibovitz culture medium, Collagenase type V, trypsin type I, bovine serum albumin (BSA), Triton X-100, 5-bromo-2'-deoxyuridine (BrdU), anti-BrdU monoclonal antibody, biotinylated goat anti-mouse IgG, diaminobenzidine tetrahydrochloride (DAB), and the antibiotic-antimycotic solution were purchased from Sigma Chemical Co. (St Louis, MO). Percoll solution was obtained from Pharmacia LKB (Uppsala, Sweden). Fetal bovine serum (FBS) was from Gibco BRL (Grand Island, NY). Plastic 6-well culture plates and 35-mm plates were from Costar (Cambridge, MA). Avidin-biotin peroxidase complex was from Vector Laboratories (Burlingame, CA). Apomorphine and thyrotropin-releasing hormone (TRH) were from ICN Inc. (Costa Mesa, CA). Frog NPY (melanostatin) was synthesized by the solid-phase method (27).

Animals

Adult male frogs (*Rana ridibunda*) of about 40 g body weight were purchased from a commercial supplier (J.R. Garcia Jardor, Mosqueiro, Ourense, Spain). Animals were maintained under running water at constant temperature (8ºC) with a 12-h light:12-h dark cycle, for at least 1 week before experiments started. Skin color adaptation was performed by keeping the animals under constant illumination on either a black or a white background for 3 weeks. The frogs were killed by decapitation between 8:00 and 9:00 a.m. and the neurointermediate lobes were dissected under a microscope. Animal manipulations were performed according to the recommendations of the local

ethical committees at our institutions and under the supervision of authorized investigators.

Isolation of melanotrope cells

Isolated melanotrope cells were obtained using a dispersion protocol as previously described (3). Briefly, for each experiment, 30-40 neurointermediate lobes from either black or white background-adapted animals were collected and enzymatically dissociated by incubation at 26ºC for 45 min in culture medium [Leibovitz medium diluted 2:3 (to adjust to *Rana ridibunda* osmolality) and supplemented with 1 mM glucose, 0.4 mM $CaCl_2$, and 1% antibiotic-antimycotic solution] containing 0.2% collagenase type V and 0.2% trypsin type I. Then, cells were incubated sequentially in the same medium supplemented with 2 mM and 1 mM EDTA for 5 min each. Afterwards, cells were mechanically dispersed using a siliconized Pasteur pipette until a homogeneous cellular suspension was obtained. The total number of cells and the cellular viability were determined by the Trypan blue exclusion test in a Neubauer chamber.

Cell culture

Dispersed melanotrope cells from black- or white-adapted animals were plated at a density of 300,000 cells/200 µl culture medium into 6-well culture plates and allowed to attach to the plate for 60 min. Subsequently, cell cultures received 1,800 µl/well culture medium supplemented with 10% FBS and 0.1% gentamicin sulfate, containing the corresponding test substances. Specifically, cultures from black-adapted frogs were incubated with 10^{-4} M apomorphine or 10^{-7} M NPY, and those from white-adapted frogs with 10^{-5} M TRH, at 26ºC for 72 h. As controls, cells from either black- or white-adapted frogs were cultured

in medium alone under the same incubation conditions than those treated with the corresponding factors. In all experimental groups, treatment was renewed daily.

For the determination of the apoptotic and proliferation rates of melanotropes in culture, 40,000 cell-aliquots from the initial cell suspension, obtained after the dispersion of intermediate lobes from either black- or white-adapted frogs, were plated in 35-mm plastic dishes. These cultures received the same experimental treatments as those described above.

Separation of melanotrope subpopulations

After the 72-h culture period in the presence or absence of the corresponding test substance, cells were detached from the plates by sequential incubation in Leibovitz medium containing 2 mM EDTA for 20 min, and 1 mM EDTA plus 0.05% trypsin for 15 min. Thereafter, the medium containing the cells was centrifuged at 750 X *g* for 5 min, and the pellets were resuspended in 1 ml Leibovitz medium. Cells recovered from the wells that had received the same experimental treatment were pooled together and washed again with Leibovitz medium by centrifugation (750 X *g* for 10 min at 4ºC). The cellular recovery rate and viability were determined for each experimental group.

A 250-µl sample of the cellular suspension from each experimental group (500,000 cells, approximately) was carefully loaded on the top of a Percoll density gradient prepared as described previously (3). In brief, the hyperbolic gradient of Percoll was prepared by mixing 6 ml of a 50% Percoll solution with 3 ml of a 15% Percoll solution at a rate of 0.25 ml/min. After centrifugation (3,000

X *g* for 25 min at 4ºC), 9 fractions (1 ml each) were collected manually, centrifuged (750 X *g* for 10 min at 4ºC), and pellets were resuspended in 200 µl fresh medium. The viability and recovery percentage of each fraction were determined for each experimental group. Cells in fraction 1 (bottom of the gradient) contained the HD or storage melanotrope cell subpopulation, whereas secretory or LD melanotropes separated in fractions 5 to 7 (3-7).

Determination of apoptosis

As indicated above, melanotrope cells were cultured in 35-mm dishes and exposed to 10^{-4} M apomorphine or 10^{-7} M NPY (for cultures from black-adapted frogs), or to 10^{-5} M TRH (for cultures from white-adapted animals). After the 72-h treatment period, medium was removed and cells were fixed with ethanol:acetic acid (3:1) as described previously for the same cell model (7). Briefly, nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI) to reveal the nuclear condensation and the chromatin fragmentation characteristic of apoptosis (28) and fluorescence was visualized in a Universal microscope (Zeiss, Jena, Germany) with a Fluar X40 objective in the epifluorescence mode (Nikon Corp., Tokyo, Japan). Quantitative evaluation of apoptotic cells by DAPI staining was performed by counting about 200 cells randomly selected in at least two plates per treatment group and experiment.

Measurement of cell proliferation

The proliferation index of melanotropes was evaluated after the 72-h culture period in the absence or presence of the test substances as described previously (7). After removal of the medium, cells were exposed to 10^{-6} M BrdU for 2 h at 26ºC, rinsed with 0.01 M PBS (pH 7.2), and then fixed in the culture

dishes overnight with Bouin's fixative. The BrdU-incorporating cells were identified immunocytochemically after sequential incubation in 5% H_2O_2 (30 min, room temperature), 2 N HCl (30 min, 37ºC), 0.01% trypsin type I in PBS (5 min, 37ºC), 1% BSA in PBS (30 min, room temperature), and an anti-BrdU monoclonal antiboby (overnight, 4ºC). Finally, cells were incubated with biotinylated goat anti-mouse IgG and avidin-biotin-peroxidase complex (1 h each, room temperature) and cell-bound peroxidase activity was revealed with DAB. The specificity of the immunoreaction was checked by omission of the primary antiserum. All controls were processed concurrently with melanotrope culture samples using identical protocols.

The immunostaining was visualized under a light microscope fitted with an X40 objective, and the proliferation rate was calculated on, at least, two plates per treatment group and experiment. In each plate, microscopic fields were randomly selected and, at least, 600 cells were examined.

Statistical analysis

Data are expressed as mean ± standard error of the number of experiments indicated in each figure. Differences in cell distribution were statistically analyzed by the Chi-square test. Two-way (for percentages of melanotrope cells) or one-way (for apoptotic and proliferation rates) analysis of variances (ANOVA), followed by a *post hoc* Duncan's test for multiple comparison, were applied to compare experimental treatments. Statistical analyses were assessed with the software Statistica for Windows (Statsoft Inc., Tulsa, OK). Differences were considered to be significant at $P < 0.05$.

RESULTS

Recovery and viability of melanotrope cells after the experimental treatments

After dispersion of intermediate lobes from black- and white-adapted frogs, the cell yields were similar [47,280 ± 4,006 (n = 9 independent experiments) and 53,760 ± 5,133 (n = 5) cells/intermediate lobe, respectively], and the viability of the cells was always over 90% in both groups of animals.

Results on the recovery of cells from the cultures by EDTA/trypsin treatment after treatment of melanotropes with the different test substances are shown in Table I. None of the treatments affected the recovery rate of cells from the plates except for apomorphine-treated cultures, which exhibited a significantly lower value than the corresponding control cultures. In contrast, subsequent separation of the resulting cells by density gradients did not affect the recovery of cells, which was similar in all the experimental groups (36.5 ± 6.1%, 49.1 ± 9.7%, 33.2 ± 9.1%, 57.8 ± 14.5%, and 48.8 ± 20.3% for black-background adaptation control, apomorphine-treated cells, NPY-treated cells, white-background adaptation control, and TRH-treated cells, respectively).

Effects of apomorphine and NPY on cultured melanotrope cells from black-background adapted frogs

Separation in Percoll density gradients of the total population of melanotrope cells from black-adapted animals after the 72-h period under control conditions yielded a bimodal distribution of cells through the gradient (Fig. 1A). Specifically, melanotropes were separated into two distinct zones of

the gradient, fraction 1 and fractions 5 to 7, which corresponded to the subpopulations of hormone-storage (HD) and secretory (LD) melanotropes, respectively, which have been previously characterized in detail (3-7). Cells separated in these fractions accounted for by 77.2% of the total number of cells recovered from the gradient, among which the number of secretory cells was approximately 6-fold higher than that of storage cells (Fig. 1B).

Exposure of dispersed intermediate lobe cells from black background-adapted frogs for 3 days to the dopamine receptor agonist apomorphine caused a significant modification of the sedimentation characteristics of the cells within the density gradient, which resulted in marked changes in the relative proportions of cells separated in each fraction (Chi-square test, $P < 0.05$; Fig. 1A). As shown in Fig. 1B, apomorphine treatment reduced by almost 20% the proportion of secretory cells with respect to control values, while it evoked a concomitant increase in the percentage of storage melanotropes, which was two-fold higher than that found under control conditions (Fig. 1B).

Similar to that observed for apomorphine, long-term *in vitro* exposure of melanotrope cells from black-adapted frogs to NPY resulted in a shift of the cell population to the heavy fractions of the gradient (Chi-square test, $P < 0.05$; Fig. 2A). Indeed, NPY increased by 24.3% the proportion of storage melanotropes and decreased by 18.5% that of secretory melanotropes when compared to the percentages obtained for the corresponding cell subpopulations from control cultures (Fig. 2B).

We also assessed the rates of proliferation and apoptosis in cultures of melanotropes from black-background adapted animals exposed to medium alone or to the inhibitors apomorphine or NPY (Table I). This revealed that

neither apomorphine nor NPY modified the apoptotic rate of melanotropes in culture when compared to control values. Similarly, no differences were observed in the proliferation index between control and NPY-treated cultures, and although apomorphine-treated cells exhibited a lower BrdU-labeling index than control cells, this difference did not reach statistical significance.

Effects of TRH on cultured melanotrope cells from white-background adapted frogs

The distributions of both control and TRH-treated cells obtained from white-background adapted animals in the density gradient are illustrated in figure 3A. After a 72-h culture period, melanotropes from both experimental groups essentially separated into the HD and LD fractions (1 and 5 to 7, respectively). Actually, the overall percentage of cells contained in both cell subsets for control and TRH-treated cultures represented 85.2% and 89.5%, respectively, of the total number of cells recovered from the gradient. However, the proportion of melanotropes separated in each density fraction was significantly different for each experimental group (Chi-square test, $P < 0.05$; Fig. 3A). Thus, whereas TRH increased the number of secretory cells (16.1%), it concurrently decreased that of storage melanotropes (11.8%) when compared to the corresponding values obtained from cells cultured in medium alone. In contrast to this effect of TRH on the balance between the two melanotrope cell subtypes, the tripeptide did not alter the rate of apoptosis or proliferation in melanotropes from white-adapted frogs in culture (Table I).

DISCUSSION

Previous studies on skin pigmentation adaptation to background color have shown that the relative proportions of the two functionally distinct cell subsets that comprise the population of melanotrope cells of the frog intermediate lobe (3-5, 29) change reciprocally in strict correspondence with the α-MSH requirements imposed by the environment (6-7). Thus, in conditions of high α-MSH demand, under a black background, the highly active melanotrope cell subset predominates over the hormone-storage melanotrope subpopulation whereas similar proportions of both melanotrope cell subtypes are present in the intermediate lobe of animals that require low plasma α-MSH levels, i.e. white-background adapted animals. The results presented in these works suggested the existence of a functional link between the adaptation process and the balanced interconversions of secretory and storage melanotropes (6-7). In the present study, we demonstrate that the hypothalamic factors NPY, dopamine and TRH play a critical role in the physiological control of intermediate lobe function through their action on the determination and maintenance of the specific functional state of melanotropes.

Involvement of dopamine in the neuroendocrine regulation of skin color adaptation in amphibians is supported by earlier studies demonstrating that agents that induce catecholamine depletion in fibers reaching the intermediate lobe caused skin darkening (for review see Ref. 15). Accordingly, our first set of studies was aimed at elucidating the effects of the major cathecolaminergic inhibitor dopamine on melanotrope cells from animals displaying high plasma α-MSH levels, that is, black-background adapted frogs. Thus, long-term *in vitro*

treatment of intermediate lobe cells from these animals, which essentially correspond to secretory or LD melanotropes (6, 7), caused a significant modification of the sedimentation characteristics of the cells within the density gradient with respect to untreated controls, which resulted in marked changes in the relative proportions of the two cell subtypes. Specifically, apomorphine reduced the percentage of secretory cells and evoked a concomitant increase in that of storage cells. Inasmuch as these changes are reminiscent of those observed during adaptation of the animals *in vivo* to a white background (6-7), our results strongly suggest that an important mechanism employed by dopamine to regulate the adaptation process would be by inducing secretory melanotropes to acquire the hormone-storage phenotype.

Since percentages of the two subtypes of melanotropes in the intermediate lobe are relative and depend on the total number of cells of the gland, we assessed the rates of proliferation and apoptosis in the cell cultures to ascertain whether apomorphine-induced changes resulted from true conversions of cells from one subpopulation (LD) into its counterpart (HD) or, alternatively, from an increase in proliferation of storage cells and/or a concomitant disappearance of secretory cells. This revealed that, in fact, the BrdU-labeling index in apomorphine-treated cultures was slightly, albeit not significantly, lower than that observed in the corresponding controls. This indicates that the net increase in the number of storage melanotropes induced by apomorphine can not be accounted for by an enhanced rate of cellular proliferation, and therefore that the cells that separate in this subpopulation after apomorphine treatment do not derive from mitosis of preexisting cells. In support of these findings, it has been shown that dopamine or its agonists

inhibit proliferation of rat melanotropes both *in vivo* and *in vitro* (30-32), as well as that of other pituitary cell types such as lactotropes (33, 34). Actually, dopamine and its agonists are commonly used in prolactinoma therapy to reduce tumor size because of their well-known antiproliferative (35) and cytotoxic (36) properties and we consequently expected apomorphine to increase apoptosis in our cultures. However, the apoptotic rate was similar in control and apomorphine-treated cultures. Nevertheless, at this point we should introduce the caveat that assessment of apoptosis might have been slightly underestimated if cells that progressed into later stages of apoptosis detached from the culture dish and were thus undetectable, as suggested by the lower cell recovery found in apomorphine-treated cultures. This caution notwithstanding, it must be emphasized that even a selective disappearance of secretory melanotropes caused by apomorphine could not account for the changes observed. Therefore, when viewed as a whole, our results strongly suggest that storage melanotropes appear as a result of the apomorphine-induced conversion of secretory cells.

We also tested the effect of long-term *in vitro* exposure of melanotrope cells from black-adapted frogs to the naturally occurring hypothalamic inhibitor NPY (27). Results from these experiments showed that NPY caused a marked decrease in the number of secretory cells and a concurrent increase in that of storage cells, without altering the rate of either cellular proliferation or apoptosis. These findings indicate that, as found for apomorphine, NPY is able to induce the transition of melanotrope cells from a highly active secretory physiological condition to a storage state. Interestingly, the relative proportions of the two subtypes of melanotropes after culture of intermediate lobe cells

from black-adapted frogs in the presence of either apomorphine or NPY were strikingly similar to those observed in intermediate lobes of white-background adapted frogs (6-7). These results support the idea that these inhibitors are able to induce *in vitro* the same cellular changes that occur *in vivo* during white-background adaptation. Moreover, they strongly suggest that dopamine and NPY play a pivotal role during this physiological process. Consistent with this notion, quantitative immunocytochemistry and *in situ* hybridization studies of the hypothalamus from black-background adapted *Xenopus* have shown that the levels of NPY mRNA and NPY-immunoreactivity are very low in the ventrolateral part of the suprachiasmatic nucleus, where NPY-producing neurons controlling intermediate lobe function are located (37-39). In contrast, high mRNA levels and NPY-immunoreactivity are found in this hypothalamic region in white-adapted toads (38, 39).

Having established that hypothalamic inhibitors can mimic *in vitro* the cellular changes that occur during white-background adaptation, we next asked whether TRH, the major hypothalamic stimulator for amphibian melanotrope cells (15, 24-26), could induce the cellular changes observed during adaptation of frogs to a black background [*i.e.* conversion of storage cells into secretory cells (6-7)]. Indeed, in the melanotrope population of white-adapted frogs, which comprises similar proportions of both cell subtypes (6-7), long-term *in vitro* exposure to TRH markedly increased the number of secretory melanotropes while it concurrently decreased that of hormone-storage cells. Inasmuch as TRH did not alter apoptosis or proliferation in these cultures, such changes in proportions are, necessarily the result of the conversion of storage cells into active melanotropes. Moreover, the percentages of both types of

melanotropes observed after treatment of intermediate lobe cells from white-adapted animals with TRH were virtually identical to those found in animals physiologically adapted to a black background (6-7). Therefore, our results demonstrate that TRH can mimic *in vitro* the effects caused by black-background adaptation on frog melanotrope cell subpopulations, and thereby strongly support the involvement of the tripeptide in the adaptation process.

The concept that the hypothalamus can control the composition of the population of a given pituitary cell type, *i.e.* the balance between its distinct subpopulations, raises two important questions. The first relates to the possible physiological relevance of such regulatory mechanism. The present results, together with those reported previously on amphibian melanotropes may provide a good example in that regard. As previously demonstrated (3-5, 29), frog storage melanotropes are heavily granulated and contain high intracellular α-MSH levels, although they show low levels of hormone biosynthesis and release under basal culture conditions. More importantly, storage melanotropes also exhibit a limited response, in terms of both POMC expression and α-MSH release, to *in vitro* short-term treatment with TRH (3-4). In addition, among the three major secretoinhibitors of the frog intermediate lobe (23), dopamine, GABA and NPY, only high concentrations of the latter are able to reduce the already low secretory activity of hormone-storage melanotropes (5, 29). In clear contrast, secretory melanotropes in culture are highly responsive in the short-term to TRH stimulation (4), as well as to the hypothalamic inhibitors dopamine, GABA, and NPY (5, 29). In view of these and our present findings, it seems reasonable to suggest that the hypothalamus controls plasma α-MSH levels during the process of adaptation to background color by regulating the total

activity of the intermediate lobe through two different, but interrelated mechanisms: 1) in the short term, by acutely controlling hormone synthesis and release, and 2) in the long term, by converting highly secretory cells into hormone-storage cells and *vice versa*. This process is graphically illustrated in Fig. 4. Thus, during white-background adaptation, dopamine and NPY would reduce α-MSH levels in the short term by inhibiting its release from the highly responsive, predominant secretory or LD subtype in black-adapted frogs. At the same time, dopamine and NPY would enable a more effective long-term reduction of α-MSH production by causing a lasting conversion of highly active and responsive secretory melanotropes into poorly responsive hormone-storage cells. Conversely, during adaptation from a white to a black background, the required high α-MSH levels would be achieved primarily by TRH in the short term through stimulation of α-MSH release from highly responsive melanotropes. Concurrently, TRH would also induce a long-term conversion of the hormone-storage cells into their active counterparts. In sum, this system would provide the hypothalamus an additional, long-lasting mechanism to finely regulate the activity of the intermediate lobe in an effective and economical manner.

The second question that derives from our findings is two-pronged: what form does the conversion of cells from one subpopulation into the other cell subtype take, and what precise factors and events do underlie this regulatory process? Regarding the first part of the question, we and others have previously suggested that heterogeneity within pituitary cell types, such as melanotropes (7), somatotropes (10), gonadotropes (8,9) and lactotropes (40), is in fact the reflection of cells undergoing a secretory cycle. This cellular

process would comprise the transition of cells from a phase or subpopulation to the other one due not only to a loss or increase in granule content, but also to changes in key functional features of the cells. In the case of melanotropes, such changes would include selective responsiveness to regulatory factors, as well as the differential regulation of the synthesis, maturation, acetylation rate, and secretion of α-MSH along with variations in other important components of the secretory pathway (41, 42). Regarding the second part of the question, the precise regulatory mechanisms that underlie the melanotrope cell secretory cycle await elucidation. One can envision a number of points at which the activity and responsiveness of melanotrope cells can be regulated. This might include from transcription factors to signaling enzymes and proteins, as well as membrane receptors. To give just an example, bromocriptine has been shown to regulate the type of G proteins (43) and dopamine receptor isoforms (44) expressed in rat melanotrope cells. Future studies should aim at ascertaining the points at which this regulation is primarily exerted, and at determining whether the secretory cycle is a general mechanism for the control of endocrine cell activity.

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REFERENCES

1. Bagnara JT, and Hadley ME Chromatophores and color changes, the comparative physiology of animal pigmentation. Englewood Cliffs, New Jersey: Prentice Hall, 1973.

2. Wilson JF, and Morgan MA. α-Melanotropin-like substances in the pituitary and plasma of *Xenopus laevis* in relation to colour change responses. *Gen Comp Endocrinol* 38: 172-182, 1979.

3. Gonzalez de Aguilar JL, Tonon MC, Ruiz-Navarro A, Vaudry H, and Gracia-Navarro F. Morphological and functional heterogeneity of frog melanotrope cells. *Neuroendocrinology* 59: 176-182, 1994.

4. Gonzalez de Aguilar JL, Malagon MM, Vazquez-Martinez RM, Lihrmann I, Tonon MC, Vaudry H, and Gracia-Navarro F. Two frog melanotrope cell subpopulations exhibiting distinct biochemical and physiological patterns in basal conditions and under thyrotropin-releasing hormone stimulation. *Endocrinology* 138: 970-977, 1997.

5. Gonzalez de Aguilar JL, Malagon MM, Vazquez-Martinez RM, Martinez-Fuentes AJ, Tonon MC, Vaudry H, and Gracia-Navarro F. Differential effects of dopamine on two frog melanotrope cell subpopulations. *Endocrinology* 140: 159-164, 1999.

6. Vazquez-Martinez RM, Malagon MM, Gonzalez de Aguilar JL, Desrues L, Tonon MC, Vaudry H, and Gracia-Navarro F. Relationship between melanotrope cell heterogeneity and background adaptation in the frog intermediate lobe. *Ann N Y Acad Sci* 839: 431-433, 1998.

7. Vazquez-Martinez R, Peinado JR, Gonzalez De Aguilar JL, Desrues L, Tonon MC, Vaudry H, Gracia-Navarro F, and Malagon MM. Melanotrope

cell plasticity: a key mechanism for the physiological adaptation to background color changes. *Endocrinology* 142: 3060-3067, 2001.

8. Torronteras R, Castano JP, Almaden Y, Ruiz-Navarro A, and Gracia-Navarro F. Hormonal storage patterns and morphological heterogeneity of porcine gonadotrope cells during postnatal development. *Mol Cell Endocrinol* 97: 51-59, 1993.
9. Torronteras R, Dobado-Berrios PM, Garcia-Navarro S, Malagon MM, and Gracia-Navarro F. Heterogeneous response of porcine gonadotrope subpopulations to gonadotropin releasing hormone (GnRH) during postnatal development. *Mol Cell Endocrinol* 112: 203-213, 1995.
10. Dobado-Berrios PM, Ruiz-Navarro A, Lopez-Pedrera R, Gonzalez de Aguilar JL, Torronteras R, Hidalgo-Diaz C, and Gracia-Navarro F. Heterogeneity of growth hormone (GH)-producing cells in aging male rats: in vitro GH releasing activity of somatotrope subpopulations. *Mol Cell Endocrinol* 123: 127-137, 1996.
11. Childs GV, Unabia G, Lee BL, and Lloyd J. Maturation of follicle-stimulating hormone gonadotropes during the rat estrous cycle. *Endocrinology* 131: 29-36, 1992.
12. Childs GV, Unabia G, Lee BL, and Rougeau D. Heightened secretion by small and medium-sized luteinizing hormone (LH) gonadotropes late in the cycle suggests contributions to the LH surge or possible paracrine interactions. *Endocrinology* 130: 345-352, 1992.
13. Childs GV, Unabia G, and Lloyd J. Recruitment and maturation of small subsets of luteinizing hormone gonadotropes during the estrous cycle. *Endocrinology* 130: 335-344, 1992.

14. Childs GV, Unabia G, and Rougeau D. Cells that express luteinizing hormone (LH) and follicle-stimulating hormone (FSH) beta-subunit messenger ribonucleic acids during the estrous cycle: the major contributors contain LH beta, FSH beta, and/or growth hormone. *Endocrinology* 134: 990-997, 1994.

15. Danger JM, Lamacz M, Leroux P, Adjeroud S, Andersen AC Verburg van Kemenade L, Jenks BG, Pelletier G, Stoeckel L, Burlet A, Kupryszewski G, and Vaudry H. Multihormonal control of melanotropin secretion in cold blooded vertebrates. In: *The Melanotropic Peptides*, edited by Hadley ME. New York: CRC Press, 1988, vol 1, pp. 127-171.

16. Verburg-Van Kemenade BM, Jenks BG, and Driessen AG. GABA and dopamine act directly on melanotropes of *Xenopus* to inhibit MSH secretion. *Brain Res Bull* 17: 697-704, 1986.

17. de Rijk EP, van Strien FJ, and Roubos EW. Demonstration of coexisting catecholamine (dopamine), amino acid (GABA), and peptide (NPY) involved in inhibition of melanotrope cell activity in *Xenopus laevis*: a quantitative ultrastructural, freeze- substitution immunocytochemical study. *J Neurosci* 12: 864-871, 1992.

18. Dotman CH, Cruijsen PM, Jenks BG, and Roubos EW. Differential action of secreto-inhibitors on proopiomelanocortin biosynthesis in the intermediate pituitary of *Xenopus laevis*. *Endocrinology* 137: 4551-4557, 1996.

19. de Rijk EP, Jenks BG, Vaudry H, and Roubos EW. GABA and neuropeptide Y co-exist in axons innervating the neurointermediate lobe

of the pituitary of *Xenopus laevis*--an immunoelectron microscopic study. *Neuroscience* 38: 495-502, 1990.

20. Danger JM, Leboulenger F, Guy J, Tonon MC, Benyamina M, Martel JC, Saint-Pierre S, Pelletier G, and Vaudry H. Neuropeptide Y in the intermediate lobe of the frog pituitary acts as an α-MSH-release inhibiting factor. *Life Sci* 39: 1183-1192, 1986.

21. Cailliez D, Danger JM, Polak JM, Pelletier G, Andersen AC, Leboulenger F, and Vaudry H. Co-distribution of neuropeptide Y and its C-terminal flanking peptide in the brain and pituitary of the frog *Rana ridibunda*. *Neurosci Lett* 74: 163-168, 1987.

22. Verburg-van Kemenade BM, Jenks BG, Danger JM, Vaudry H, Pelletier G, and Saint-Pierre S. An NPY-like peptide may function as MSH-release inhibiting factor in *Xenopus laevis*. *Peptides* 8: 61-67, 1987.

23. Tonon MC, Bosler O, Stoeckel ME, Pelletier G, Tappaz M, and Vaudry H. Co-localization of tyrosine hydroxylase, GABA and neuropeptide Y within axon terminals innervating the intermediate lobe of the frog *Rana ridibunda*. *J Comp Neurol* 319: 599-605, 1992.

24. Lamacz M, Hindelang C, Tonon MC, Vaudry H, and Stoeckel ME. Three distinct thyrotropin-releasing hormone-immunoreactive axonal systems project in the median eminence-pituitary complex of the frog *Rana ridibunda*. Immunocytochemical evidence for co-localization of thyrotropin-releasing hormone and mesotocin in fibers innervating pars intermedia cells. *Neuroscience* 32: 451-462, 1989.

25. Verburg-van Kemenade BM, Jenks BG, Visser TJ, Tonon MC, and Vaudry H. Assessment of TRH as a potential MSH release stimulating factor in *Xenopus laevis*. *Peptides* 8: 69-76, 1987.

26. Dotman CH, Maia A, Jenks BG, and Roubos EW. Sauvagine and TRH differentially stimulate proopiomelanocortin biosynthesis in the *Xenopus laevis* intermediate pituitary. *Neuroendocrinology* 66: 106-113, 1997.

27. Chartrel N, Conlon JM, Danger JM, Fournier A, Tonon MC, and Vaudry H. Characterization of melanotropin-release-inhibiting factor (melanostatin) from frog brain: homology with human neuropeptide Y. *Proc Natl Acad Sci USA* 88: 3862-3866, 1991.

28. Collins JA, Schandi CA, Young KK, Vesely J, and Willingham MC. Major DNA fragmentation is a late event in apoptosis. *J Histochem Cytochem* 45: 923-934, 1997.

29. Vazquez-Martinez R, Malagon MM, Castaño JP, Tonon MC, Vaudry H, and Gracia-Navarro F. Amphibian melanotrope subpopulations respond differentially to hypothalamic secreto-inhibitors. *Neuroendocrinology* 73: 426-434, 2001.

30. Chronwall BM, Millington WR, Griffin WS, Unnerstall JR, and O'Donohue TL. Histological evaluation of the dopaminergic regulation of proopiomelanocortin gene expression in the intermediate lobe of the rat pituitary, involving *in situ* hybridization and [^{3}H]thymidine uptake measurement. *Endocrinology* 120: 1201-1211, 1987.

31. Gehlert DR, Bishop JF, Schafer MP, and Chronwall BM. Rat intermediate lobe in culture: dopaminergic regulation of POMC biosynthesis and cell proliferation. *Peptides* 9: 161-168, 1988.

32. Gary KA, and Chronwall BM. The onset of dopaminergic innervation during ontogeny decreases melanotrope proliferation in the intermediate lobe of the rat pituitary. *Int J Dev Neurosci* 10: 131-142, 1992.

33. Saiardi A, Bozzi Y, Baik JH, and Borrelli E. Antiproliferative role of dopamine: loss of D2 receptors causes hormonal dysfunction and pituitary hyperplasia. *Neuron* 19: 115-26, 1997.

34. Arita J, Hashi A, Hoshi K, Mazawa S, and Suzuki S. D2 dopamine-receptor-mediated inhibition of proliferation of rat lactotropes in culture is accompanied by changes in cell shape. *Neuroendocrinology* 68: 163-171, 1998.

35. Thorner MO, Evans WS, MacLeod RM, Nunley WC Jr, Rogol AD, Morris JL, and Besser GM. Hyperprolactinemia: current concepts of management including medical therapy with bromocriptine. *Adv Biochem Psychopharmacol* 23: 165-189, 1980.

36. Tindall GT, Kovacs K, Horvath E, and Thorner MO. Human prolactin-producing adenomas and bromocriptine: a histological, immunocytochemical, ultrastructural, and morphometric study. *J Clin Endocrinol Metab* 55: 1178-1183, 1982.

37. Tuinhof R, Laurent FY, Ebbers RG, Smeets WJ, Van Riel MC, and Roubos EW. Immunocytochemistry and in situ hybridization of neuropeptide Y in the hypothalamus of *Xenopus laevis* in relation to background adaptation. *Neuroscience* 55: 667-675, 1993.

38. Kramer BM, Welting J, Berghs CA, Jenks BG, and Roubos EW. Functional organization of the suprachiasmatic nucleus of *Xenopus*

laevis in relation to background adaptation. *J Comp Neurol* 432: 346-355, 2001.

39. Kramer BM, Song JY, Westphal NJ, Jenks BG, and Roubos EW. Regulation of neurons in the suprachiasmatic nucleus of *Xenopus laevis*. *Comp Biochem Physiol B Biochem Mol Biol* 132: 269-274, 2002.

40. Kukstas LA, Verrier D, Zhang J, Chen C, Israel JM, and Vincent JD. Evidence for a relationship between lactotroph heterogeneity and physiological context. *Neurosci Lett* 120: 84-86, 1990.

41. Gracia-Navarro F, Malagon MM, Castano JP, Garcia-Navarro S, Sanchez-Hormigo A, Luque RM, Peinado JR, and Delgado E. Secretory plasticity of pituitary cells: a mechanism of hormonal regulation. *Arch Physiol Biochem* 110: 106-112, 2002.

42. Peinado JR, Castano JP, Vazquez-Martinez R, Anouar Y, Tonon MC, Vaudry H, Gracia-Navarro F, and Malagon MM. Amphibian melanotrophs as a model to analyze the secretory plasticity of endocrine cells. *Gen Comp Endocrinol* 126: 4-6, 2002.

43. Sands SA, Dickerson DS, Morris SJ, and Chronwall BM. Dopamine D2 receptor stimulation alters G-protein expression in rat pituitary intermediate lobe melanotropes. *Endocrine* 6: 325-333, 1997.

44. Chronwall BM, Sands SA, Dickerson DS, Sibley DR, and Gary KA. Melanotrope dopamine D2 receptor isoform expression in the developing rat pituitary. *Int J Dev Neurosci* 14: 77-86, 1996.

Figure Legends

FIG 1: (A) Distribution through the Percoll density gradient of melanotrope cells from black-background adapted frogs cultured in medium alone (solid bars) or in the presence of 10^{-4} M apomorphine (open bars) for 3 days. Data are expressed as mean ± SE of 5 independent experiments. Statistical differences were assesed by Chi-square test, $P < 0.05$ *vs.* control cultures. (B) Percentage of cells recovered in the low density fractions of the gradient (LD, fractions 5-7), corresponding to *secretory melanotropes*, and in the high density fraction (HD, fraction 1), corresponding to *hormone-storage melanotropes*, from the intermediate lobe of black-background adapted frogs cultured for 3 days in the absence (solid bars) or in the presence of 10^{-4} M apomorphine (open bars). The data represent the mean ± SE of 5 independent experiments. Statistical differences were assessed by two-way ANOVA followed by the Multiple Comparison Duncan's test. *; $P < 0.05$ *vs.* control cultures.

FIG 2: (A) Distribution through the Percoll density gradient of melanotrope cells from black-background adapted frogs cultured in medium alone (solid bars) or in the presence of 10^{-7} M NPY (open bars) for 3 days. Data are expressed as mean ± SE of 5 independent experiments. Statistical differences were assesed by Chi-square test, $P < 0.05$ *vs.* control cultures. (B) Percentage of cells recovered in fractions 5-7 of the gradient (low density –LD- or secretory melanotropes), and in fraction 1 (high density –HD- or hormone-storage melanotropes), from the intermediate lobe of black-background adapted frogs cultured for 3 days in the absence (solid bars) or in the presence of 10^{-7} M NPY (open bars). The data represent the mean ± SE of 5 independent experiments.

Statistical differences were assessed by two-way ANOVA followed by the Multiple Comparison Duncan's test. *; $P < 0.05$ *vs.* control cultures.

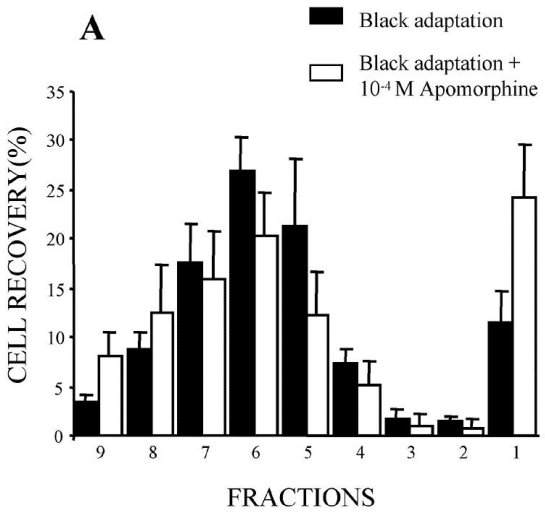
FIG 3: (A) Distribution through the Percoll density gradient of melanotrope cells from white-background adapted frogs cultured in medium alone (solid bars) or in the presence of 10^{-5} M TRH (open bars) for 3 days. Data are expressed as mean ± SE of 3 independent experiments. Statistical differences were assesed by Chi-square test, $P < 0.05$ *vs.* control cultures. (B) Percentage of cells recovered in fraction 1 and fractions 5-7, corresponding to hormone-storage (HD cells) and secretory melanotropes (LD cells), respectively, from the intermediate lobe of white-background adapted frogs cultured for 3 days in the absence (solid bars) or in the presence of 10^{-5} M TRH (open bars). The data represent the mean ± SE of 3 independent experiments. Statistical differences were assessed by two-way ANOVA followed by the Multiple Comparison Duncan's test. *; $P < 0.05$ *vs.* control cultures.

FIG 4: Representation of the secretory cycle exhibited by frog melanotrope cells. Black-background adaptation (black arrow) would induce the conversion of *hormone-storage melanotropes* into highly active *secretory melanotropes via* an increase in stimulatory hypothalamic inputs (*i.e.* enhanced TRH secretion and, likely, blockade of inhibitory inputs), in order to increase hormone production. Conversely, during white adaptation (open arrow), in which a lower amount of hormone is required, an inverse process would occur, mediated by an increase in inhibitory hypothalamic inputs (*i.e.* increased dopamine and NPY release). Small vesicles represent the secretory granule content; undulating lines indicate the amount of the α-MSH precursor transcripts; and gray arrows represent the secretory activity of α-MSH.

Table I: Apoptotic and proliferation rates in cultures of intermediate lobe cells from black- and white-adapted frogs after a 3-day culture period in the absence (control) or the presence of various test substances.

	Black-adapted frogs			**White-adapted frogs**	
	Control	Apomorphine (10^{-4} M)	NPY (10^{-7} M)	Control	TRH (10^{-5} M)
Cell recovery after culture (%)	51.6 ± 7.4	28.8 ± 5.2*	44.3 ± 9.7	56.0 ± 6.8	51.8 ± 9.7
Apoptotic index (%)	2.55 ± 0.71	1.30 ± 0.19	2.85 ± 1.52	2.56 ± 1.00	1.55 ± 0.36
Proliferation rate (%)	0.87 ± 0.32	0.10 ± 0.14	0.80 ± 0.25	0.73 ± 0.59	0.60 ± 0.12

Data are expressed as percentages and represent the mean ± SE of 3 independent experiments. Statistical differences were analyzed by one-way ANOVA followed by the Multiple Comparison Duncan's test. *; $P < 0.05$ *vs.* corresponding control.


A
Black adaptation
Black adaptation +
10-4 M Apomorphine
CELL RECOVERY(%)
35
30
25
20
15
10
5
0
9
8
7
6
5
4
3
2
1
FRACTIONS

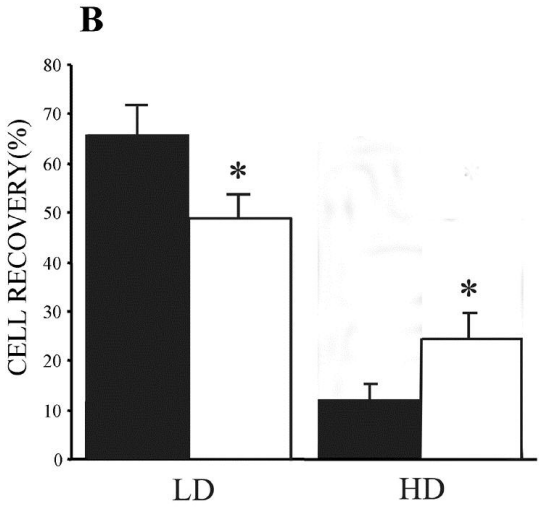

B
CELL RECOVERY(%)
80
70
60
50
40
30
20
10
0
*
*
LD
HD

Fig. 1

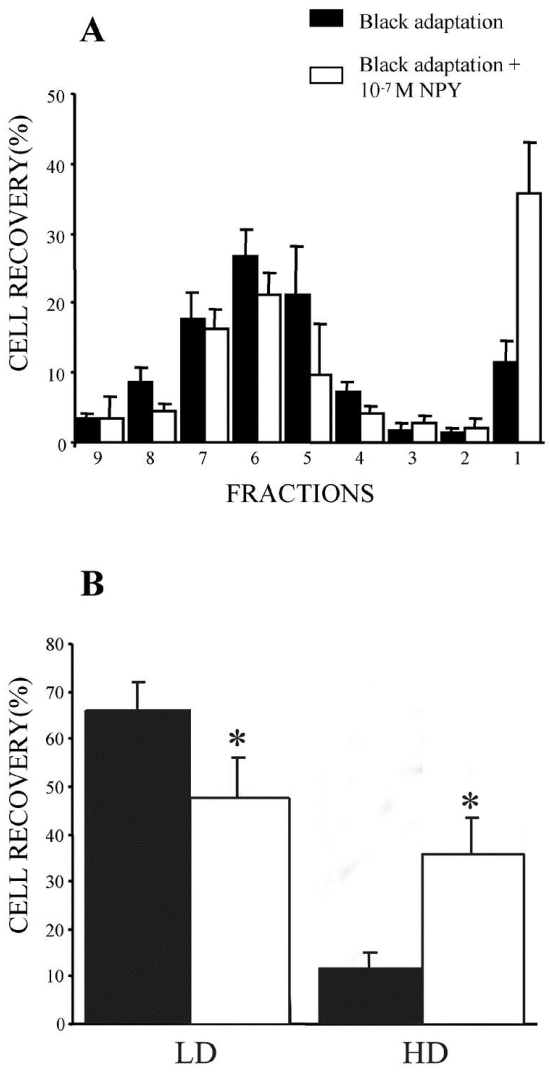

A
Black adaptation
Black adaptation +
10-7 M NPY
CELL RECOVERY(%)
50
40
30
20
10
0
9
8
7
6
5
4
3
2
1
FRACTIONS
B
CELL RECOVERY(%)
80
70
60
50
40
30
20
10
0
*
*
LD
HD

Fig. 2

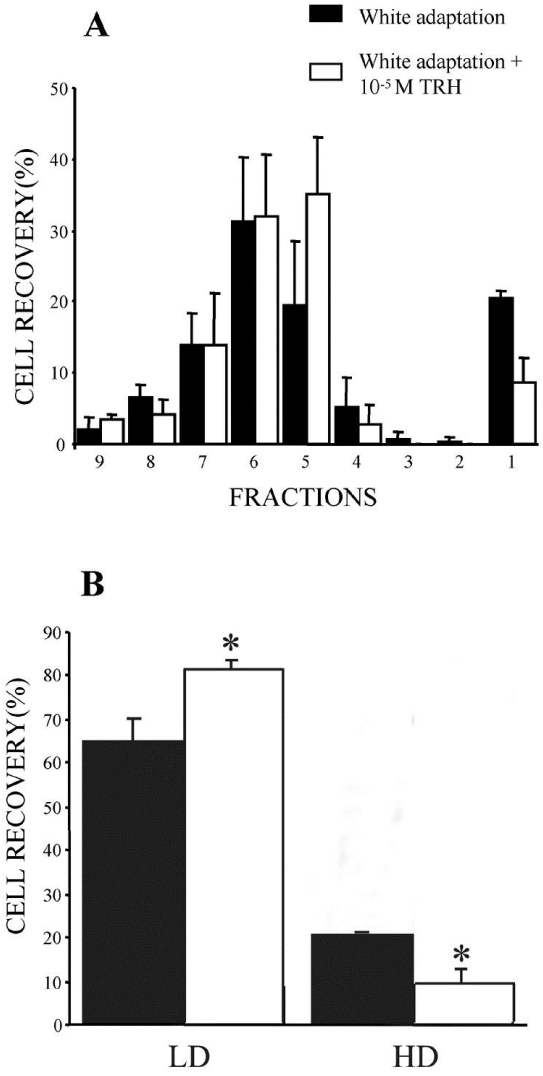

A
White adaptation
White adaptation +
10-5 M TRH
CELL RECOVERY(%)
50
40
30
20
10
0
9
8
7
6
5
4
3
2
1
FRACTIONS
B
CELL RECOVERY(%)
90
80
70
60
50
40
30
20
10
0
*
*
LD
HD

Fig. 3

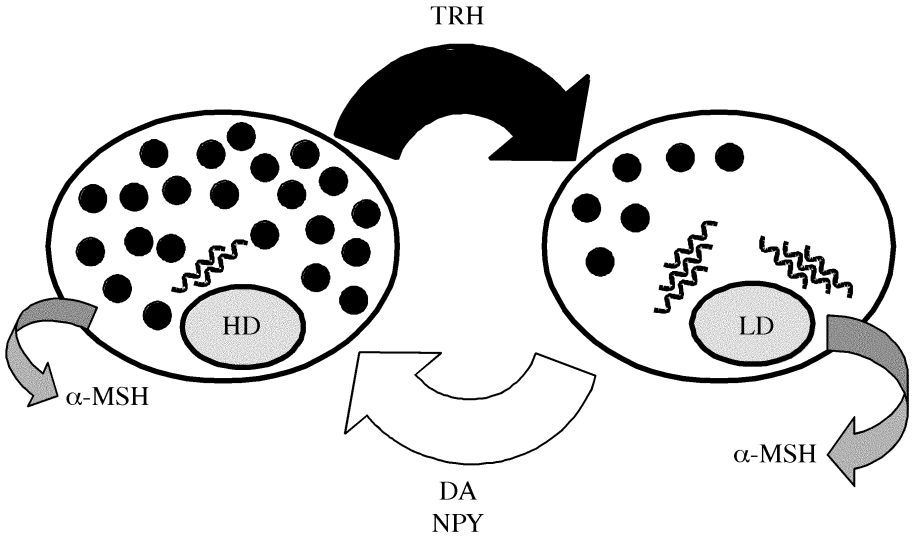


Fig. 4