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Novel Pituitary Actions of TAC3 Gene Products in Fish Model: - Receptor Specificity and Signal Transduction for Prolactin and Somatolactin α Regulation by Neurokinin B (NKB) and NKB-Related Peptide in Carp Pituitary Cells.

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Abstract:	TAC3 is a member of tachykinins and its gene product neurokinin B (NKB) has recently emerged as a key regulator for luteinizing hormone (LH) through modulation of kisspeptin/GnRH system within the hypothalamus. In fish models, TAC3 not only encodes NKB but also a novel tachykinin-like peptide called NKB-related peptide (NKBRP) and the pituitary actions of these TAC3 gene products are still unknown. Using grass carp as a model, the direct effects and post-receptor signaling for the two TAC3 products were examined at the pituitary level. Grass carp TAC3 was cloned and confirmed to encode NKB and NKBRP similar that of other fish species. In grass carp pituitary cells, NKB and NKBRP treatment did not affect LH release and gene expression but up-regulated prolactin (PRL) and somatolactin α (SLα) secretion, protein production and transcript expression. The stimulation by these two TAC3 gene products on PRL and SLα release and mRNA levels were mediated by pituitary NK2 and NK3 receptors, respectively. Apparently, NKB- and NKBRP-induced SLα secretion and transcript expression were caused by AC/cAMP/PKA, PLC/IP3/PKC and Ca2+/CaM/CaMK-II activation. The signal transduction mechanisms for the corresponding effects on PRL release and gene expression were also similar, except that the PKC component was not involved. These findings suggest that the two TAC3 gene products do not play a role in LH regulation at the pituitary level in carp species but may serve as novel stimulators for PRL and SLα synthesis and secretion through overlapping post-receptor signaling mechanisms coupled to NK2 and NK3 receptors, respectively.

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Novel Pituitary Actions of TAC3 Gene Products in Fish Model: - Receptor Specificity and Signal Transduction for Prolactin and Somatolactin α Regulation by Neurokinin B (NKB) and NKB-Related Peptide in Carp Pituitary Cells.

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Page Heading: Pituitary actions of TAC3 gene products

Precis: First demonstration of pituitary regulation of prolactin and somatolactin α by TAC3 gene products via overlapping signaling mechanisms coupled to NK2 and NK3 receptors, respectively.

Key Words: Neurokinin B; NKB-related Peptide; NK2 receptor; NK3 receptor; Signal Transduction

Abbreviations: NKB, Neurokinin B; NKBRP, NKB-related peptide; NK2R, Type 2 NK receptor; NK3R; Type 3 NK receptor; SL, Somatolactin; PRL, Prolactin; PKC, Protein kinase C; PKA, Protein kinase A; $[Ca^{2+}]i$, Intracellular Ca^{2+}; $[Ca^{2+}]e$, Extracellular Ca^{2+}; AC, Adenylyl cyclase; PLC, phospholipase C; IP_3, Inositol 1,4,5-triphosphate; VSCC, Voltage-sensitive calcium channel; CaM, Calmodulin; CaMK-II, Ca^{2+}/CaM-dependent protein kinase II.

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Abstract

TAC3 is a member of tachykinins and its gene product neurokinin B (NKB) has recently emerged as a key regulator for luteinizing hormone (LH) through modulation of kisspeptin/GnRH system within the hypothalamus. In fish models, TAC3 not only encodes NKB but also a novel tachykinin-like peptide called NKB-related peptide (NKBRP) and the pituitary actions of these TAC3 gene products are still unknown. Using grass carp as a model, the direct effects and post-receptor signaling for the two TAC3 products were examined at the pituitary level. Grass carp TAC3 was cloned and confirmed to encode NKB and NKBRP similar to that of other fish species. In carp pituitary cells, NKB and NKBRP treatment did not affect LH release and gene expression but up-regulated prolactin (PRL) and somatolactin α (SLα) secretion, protein production and transcript expression. The stimulation by these two TAC3 gene products on PRL and SLα release and mRNA levels were mediated by pituitary NK2 and NK3 receptors, respectively. Apparently, NKB- and NKBRP-induced SLα secretion and transcript expression were caused by AC/cAMP/PKA, PLC/IP3/PKC and Ca^{2+}/CaM/CaMK-II activation. The signal transduction for the corresponding responses on PRL release and mRNA expression were also similar, except that the PKC component was not involved. These findings suggest that the two TAC3 gene products do not play a role in LH regulation at the pituitary level in carp species but may serve as novel stimulators for PRL and SLα synthesis and secretion via overlapping post-receptor signaling mechanisms coupled to NK2 and NK3 receptors, respectively.

(249 words)

Introduction

Tachykinins including substance P (SP), neurokinin A (NKA), neurokinin B (NKB), hemokinin-1 (HK-1) and endokinins constitute the largest group of neuropeptides in mammals. They are widely expressed at the tissue level, functionally involved in vasodilation, gut motility, nociception, immuno-modulation and neuroendocrine regulation (1), and have been implicated in clinical cases of asthma, chronic pain, inflammatory bowel syndrome, Alzheimer's disease, anxiety attack and depression (2). Multiple genes for tachykinins, e.g., TAC1 coding for SP and NKA, TAC3 coding for NKB and TAC4 coding for HK-1/endokinins, have been identified (3) and believed to be the result of gene duplication occurred during vertebrate evolution (4). The biological actions of tachykinins are mediated by three major types of neurokinin receptors (NKR), namely NK1R, NK2R and NK3R (3), which are class I G-protein coupled receptors functionally coupled with PLC/IP_3/PKC, MAPK, cAMP/PKA and Ca^{2+}-dependent cascades (5-10). Individual NKR subtypes are known to exhibit differential binding for different tachykinins, with NK1R preferring SP, NK2R preferring NKA and NK3R preferring NKB respectively (3). With potential applications in clinical treatment, structure-activity relationship for ligand/receptor interaction and development of agonists/antagonists with NKR subtype selectivity have been a major focus of tachykinin research, particularly for rational design of novel therapeutics (11).

Recently, the gene product of TAC3, namely NKB, has emerged as a key regulator for reproductive functions, especially for GnRH pusatility (12), steroid feedback (13) and puberty onset (14). The idea was first initiated by the findings that NKB and NK3R mutations can lead to hypogonadotropic hypo-gonadism and infertility in humans (15, 16) and impairment of the NKB/NK3R system can postpone puberty in animal models (e.g., delaying vaginal opening in mouse) (14). Other studies also reveal that the Kisspeptin neurons with co-expression of NKB and Dynorphin (also called "KNDy neurons") located in the arcuate nucleus (ARC) of the hypothalamus not only represent a major target for steroid negative feedback (17) but also a critical component of GnRH pulse generator regulating luteinizing hormone (LH) secretion (e.g., sheep) (18). Apparently, these neurons form an autosynaptic feedback

within the ARC with NKB-induced kisspeptin release via NK3R to trigger GnRH secretion in the hypothalamus (19, 20). NKB activation of kisspeptin output to GnRH neurons, however, can be suppressed by local release of dynorphin from KNDy neurons and this inhibition is mediated via κ-type opioid receptor (21) and highly dependent on steroid background of the animal (19). Although NKB is involved in LH regulation via kisspeptin/GnRH modulation in the hypothalamus, its pituitary actions cannot be excluded as NKR expression (e.g., NK1R & NK2R) can be detected in the pituitary (22, 23) and NKB-induced prolactin (PRL) release (24) and enhancement of TRH-induced PRL gene transcription (25) have been reported in rat pituitary cells and lactotroph cell line, respectively. Of note, NK3R has not been identified at the pituitary level in mammals and the post-receptor signaling for the pituitary actions of NKB are still unknown.

NKB regulation of reproductive functions has been recently extended to fish models. In zebrafish, NKB/NK3R system has been identified (26) and NKB treatment can also elevate plasma LH levels (27). Interestingly, the TAC3 gene in fish species not only encodes NKB but also a novel tachykinin called NKB-related peptide (NKBRP/neurokinin F) (26, 27). Similar to NKB, NKBRP was effective in activating NK3R (28) and inducing LH release in zebrafish (27). However, neuroanatomical studies in zebrafish also reveal that NKB and kisspeptin are expressed in separate neuronal populations in brain areas relevant to reproduction (26), suggesting that the "KNDy" system in fish may be different from that of mammals. In this study, the pituitary actions of NKB and the novel peptide NKBRP were examined in grass carp, a commercial fish in Asian countries with high market value. Grass carp TAC3 was cloned and its tissue expression, especially in the brain-pituitary axis, was characterized. Using primary culture of carp pituitary cells as a model, we have demonstrated for the first time that the gene products of TAC3, namely NKB and NKBRP, did not alter LH release/gene expression at the pituitary level but rather serve as novel regulators for PRL and somatolactin α (SLα) synthesis and secretion via overlapping post-receptor signaling mechanisms coupled to pituitary NK2R and NK3R, respectively.

Materials and Methods

Animal and test substances

One-year-old grass carp (*Ctenopharyngodon idellus*) with body weight of 2.0-2.5 kg were acquired from local markets and maintained in 250-liter aquaria under 12D:12L photoperiod at 20 ^{o}C. Since sexual dimorphism was not apparent in these fish, carps of mixed sexes were used for pituitary cell preparation according to the protocol approved by the committee for animal use at University of Hong Kong. Carp NKB and NKBRP were synthesized by GenScript (Piscataway, NJ). GR64349, Senktide, HK-1, L-732138, GR159897 and SB222200 were purchased from Tocris (Bristol, UK). Forskolin, H89, MDL12330A, 8-bromo-cAMP (8Br.cAMP), IBMX, 2-APB, U73122, GF109203X, Nifedipine, A23187, KN62 and Calmidazolium were obtained from Calbiochem (San Diego, CA). Test substances were prepared as 10 mM frozen stocks in small aliquots and diluted with pre-warmed culture medium to appropriate concentrations 15 min prior to drug treatment.

Cloning, copy number and tissue expression of carp TAC3

Total RNA was extracted from carp hypothalamus using Trizol (Invitrogen, Grand Island, NY) and reversely transcribed with Superscript-II (Invitrogen). 5'/3'RACE were performed to isolate the carp TAC3 cDNA using primers designed based on the conserved regions of zebrafish TAC3. Sequence alignment and phylogenetic analysis of carp TAC3 were conducted using MacVector and MEGA 6.0 (http://www.megasoftware.net/). To determine the copy number of TAC3 gene, Southern blot was performed in genomic DNA isolated from carp whole blood (29) using a DIG-labeled cDNA probe for carp TAC3. For tissue expression of TAC3 in grass carp, RT-PCR was conducted in RNA isolated from selected tissues and brain areas (30) using primers specific for carp TAC3 (see Fig.1 legend for primer sequences & PCR conditions). In these experiments, RT-PCR for β-actin was also performed as an internal control.

PRL and SLα secretion, cell content and mRNA expression

Grass carp pituitary cells prepared by trypsin/DNase digestion method (31) were seeded in 24-well plates at ~2.5×10^6 cells/ml/well and incubated with test substances for the duration as indicated. After that, culture medium was harvested for monitoring PRL and SLα release and cell lysate was prepared from pituitary cells (32) for measurement of cell content for the respective hormones. PRL and SLα levels in these samples were quantified using RIA for PRL (33) and ELISA for SLα (34) with antisera raised against the respective hormones in carp species. Total production of PRL and SLα in individual wells were deduced *pro rata* based on the protein data for cell content and secretion for the respective hormones. In parallel experiments, total RNA was isolated from pituitary cells, reversely transcribed, and subjected to quantitative PCR for grass carp PRL and SLα mRNA using a RotorGene-Q Real-time PCR system (Qiagen, Vaoencia, CA) (see Fig.2 legend for primer sequences & PCR conditions). In these PCR assays, serial dilutions of plasmid DNA with PRL or SLα ORF sequences were used as the standards for data calibration and parallel real-time PCR for β-actin was also conducted as the internal control. To examine the possible coupling of NKB/NKBRP with various signaling targets, the cell lysate prepared was also subjected to Western blot using antibodies for the phosphorylated form and total protein of $MEK_{1/2}$ (1:1,500), $ERK_{1/2}$ (1:5,000), Akt (1:1,500) and CREB (1:2,000), respectively (32, 47). (See antibody table submitted for the details.)

In situ hybridization of NK2R and NK3R in carp pituitary sections

In situ hybridization was performed in consecutive carp pituitary sections (5 μm thick) prefixed in 4% paraformaldehyde as described previously (29) using DIG-labeled antisense riboprobes for carp NK2R and NK3R, respectively. Parallel hybridization with the corresponding sense-strand riboprobes was used as the negative control. In carp pituitary sections, zonal distribution of the major cell types was revealed by in situ hybridization using double-strand DIG-labeled cDNA probes for carp PRL, GH, LHβ and SLα, respectively. In this case, hybridization without adding cDNA probes was used as the control.

RT-PCR for NKR expression in immuno-identified pituitary cells

Carp pituitary cells were spread evenly onto glass slides (~5×10^4 cells/0.5 ml/slide), fixed in Bouin's fixative and subjected to immunostaining with antisera for carp PRL (1:100,000), GH (1:50,000), SLα (1:100,000) and SLβ (1:100,000), respectively, using a Vectastain ABC Kit (Vector Lab, Burlingame, CA). After that, immno-identified PRL cells, GH cells, SLα and SLβ cells were isolated separately by laser capture microdissection (LCM) using a PixCell-II Cell Isolation System (Arcturus, MountView, CA) (29). Total RNA was extracted from individual cell types and reversely transcribed for PCR detection of grass carp NK1R (GenBank no: JQ254914), NK2R (GenBank no: JN105350) and NK3R (GenBank no: JN105350) using primers specific for the respective receptor subtypes (see Fig.3 legend for primer sequences & PCR conditions). Parallel RT-PCR for β-actin was also performed to serve as the internal control.

cAMP production and Ca^{2+} measurement in carp pituitary cells

Pituitary cells were cultured at ~3×10^6 cells/2 ml/35 mm dish and challenged with NKB/NKBRP in the presence of the phosphodiesterase inhibitor IBMX (0.1 mM). After treatment, cAMP production was quantified using a BioTrak [^{125}I]cAMP RIA Kit (Amersham, Piscataway, NJ) (30). For single-cell Ca^{2+} imaging, pituitary cells were seeded onto coverslip (~0.5×10^6 cells/ml/coverslip), pre-loaded with the Ca^{2+}-sensitive dye Fura-2/AM (5 μM, Molecular Probes, Eugene, Oregon), and tested for Ca^{2+} responses with drug treatment using a PTI DeltaScan Epifluorescence System (Photon Technology International, West Sussex, UK) (35). Ca^{2+} signals were expressed as a ratio of fluorescence emission at 510 nm obtained with excitation at 340 and 380 nm, respectively (as "F340/F380 Ratio").

Data transformation and statistics

For PRL and SLα measurement, standard curves with detectable range from 0.98 to 500 ng/ml and

ED_{50} values of 8-15 ng/ml (for PRL) and 60-80 ng/ml (for SLα) were used for data calibration with four-parameter logistic regression model of Prism 6.0 (GraphPad, San Diego, CA). For real-time PCR of PRL and SLα mRNA, standard curves with dynamic range of 10^5 and correlation coefficient ≥0.95 were used for data calibration with RotorGene-Q software 1.7 (Qiagen). Since no significant changes were noted for β-actin mRNA in our studies, PRL and SLα mRNA data as well as the corresponding protein data were simply transformed as a percentage of the mean value in the control group without drug treatment (as "%Ctrl"). The data presented (as Mean ± SEM) were pooled results from 6-8 experiments and analyzed with ANOVA followed by Dunnett's test using Prism 6.0 and differences between groups were considered as significant at $P<0.05$.

Results

Cloning and sequence analysis of grass carp TAC3

Using 5'/3'RACE, a full-length grass carp TAC3 cDNA (GenBank no: JN105351) was cloned and found to be 631 bp in size with a 91 bp 5'UTR, 378 bp ORF encoding a 126 a.a. TAC3 precursor, and 173 bp 3'UTR with two putative polyadenylation signals (Supplemental Fig.1). Although the deduced a.a. sequence of carp TAC3 precursor is only 20-23% homologous to that of mammalian counterparts, the regions for signal peptide and NKB mature peptide are highly conserved among vertebrate species (Fig.1A). Similar to other fish models, the a.a. sequence of NKBRP flanked by two dibasic cleavage sites (KR & GRR) similar to that of NKB and with a tachykinin signature motif "FXGLM" in its C-terminal can also be identified in the carp TAC3 precursor. Phylogenetic analysis based on nucleotide sequences further confirms that the newly cloned cDNA can be clustered in the clade of fish TAC3 and is closely related to TAC3a reported in zebrafish (Fig.1B).

Copy number and tissue expression of TAC3 gene

Using Southern blot, a single band hybridized with a DIG-labeled probe for TAC3 was consistently detected in carp genomic DNA with prior digestion by Pvu II, Sty I, Hind III, Pst I, EcoR V and Hinc II respectively (Fig.1C), implying that the newly cloned TAC3 is a single copy gene in carp genome. RT-PCR also revealed that, except for the spleen, TAC3 gene was ubiquitously expressed in various tissues and brain areas (Fig.1D). High levels of TAC3 expression were located in the brain, intestine and gonad, to a lower extent in the liver and gills, and with low levels in the heart, kidney and muscle. In the brain, high levels of TAC3 expression were noted in the hypothalamus and olfactory bulb, and with low levels of signals in the telencephalon, optic tectum, pituitary, cerebellum, medulla oblongata and spinal cord.

Pituitary hormone regulation by NKB and NKBRP

To examine the pituitary actions of TAC3 gene products, carp NKB and NKBRP were synthesized and tested in primary culture of carp pituitary cells. In our initial study, 24-hr incubation with NKB or NKBRP (100 nM) were able to elevate PRL and SLα mRNA levels without altering GH, LHβ, FSHβ, GtHα, TSHβ, SLβ and POMC transcript expression (Supplemental Fig.2A). Time-course experiments also revealed that NKB and NKBRP (1 μM) could increase SLα and PRL secretion, cell content and total production up to 24 hr (Fig.2A) with parallel rises in SLα and PRL mRNA levels (Fig.2B). A transient drop in PRL cell content was noted during the first 1-6 hr of NKB/NKBRP treatment, which might be the result of temporary depletion of cellular PRL stores caused by the noticeable increase in PRL secretion during the same period. In dose-dependence studies, 24-hr incubation with increasing levels of NKB or NKBRP (0.1-1000 nM) also triggered SLα and PRL release and mRNA expression in a dose-related fashion (Fig.2C). However, the treatment had no effects on transcript levels of other pituitary hormones (Supplemental Fig.2B) or altering LH, GH and SLβ release in carp pituitary cells (Supplemental Fig.2C).

Receptor specificity for SLα and PRL regulation by TAC3 gene products

As shown in Fig.3A and 3B, 24-hr treatment with NKB/NKBRP (100 nM) could up-regulate SLα and PRL release and mRNA levels in carp pituitary cells. The stimulatory effects on SLα secretion and gene expression, however, were blocked by simultaneous incubation with the NK3R antagonist SB222200 (1 μM) but not NK1R antagonist L732138 (1 μM) or NK2R antagonist GR159897 (1 μM). For the corresponding PRL responses, the stimulation by NKB and NKBRP were abrogated only by co-treatment with the NK2R antagonist GR159897. Consistent with these results, the dose-dependence of NKB/NKBRP-induced SLα mRNA expression, especially in the lower nanomolar range (0.1-10 nM), was mimicked by increasing levels of the NK3R agonist senktide but not NK1R agonist HK-1 or NK2R agonist GR64349 (Fig.3C). In the same study, the corresponding PRL mRNA data revealed a similar stimulation in 0.1-10 nM range only by the NK2R agonist GR64349 but not the other NKR agonists. Nevertheless, significant induction by high levels (up to 1 μM) of HK-1/GR64349 on SLα and HK-1/senktide on PRL mRNA expression could still be noted, presumably due to receptor cross-reactivity by high doses of NKR agonists. Similar to the gene expression responses, specific induction of SLα secretion by senktide but not GR64349 or HK-1 and PRL secretion by GR64349 but not HK-1 or senktide could be detected by 24-hr incubation with NKR agonists fixed at 10 nM level (Fig.3D).

Using in situ hybridization, zonal distribution of pituitary cells with PRL cells located in the rostral pars distalis (RPD), GH and LH cells located in proximal pars distalis (PPD) and SLα cells located in the neurointermediate lobe (NIL) could be demonstrated in the carp pituitary (Supplemental Fig.3A). Interestingly, hybridization signals for NK2R were found to overlap with the distribution of PRL cells within the RPD (Supplemental Fig.3B) whereas the signals for NK3R could be mapped to SLα cells within the NIL (Supplemental Fig.3C). To further confirm the cell-type specificity of NK2R and NK3R expression, RT-PCR of the three NKR subtypes was performed in pure populations of carp GH cells, PRL cells, SLα cells and SLβ cells isolated by LCM technique (Fig.3E). Although the PCR signals for NK1R, NK2R and NK3R were all detected in mixed populations of carp pituitary cells, NK2R signal was noted only in PRL cells while NK3R signal was found only in SLα cells. The absence of NKR signals in other cell types could not be due to RNA degradation as the PCR signals for β-actin were consistently detected in all the samples examined.

Signal transduction for SLα and PRL regulation by TAC3 gene products

As shown in Fig.4A, cAMP production in carp pituitary cells could be elevated dose-dependently by 20-min treatment with NKB and NKBRP, respectively. Besides, 24-hr incubation with the membrane-permeant cAMP analog 8Br.cAMP (10-1000 μM) and adenylate cyclase (AC) activator forskolin (1 μM) were both effective in up-regulating SLα and PRL mRNA levels (Fig.4B). Consistent with these findings, co-treatment with the AC inhibitor MDL12330A (20 μM) or PKA inhibitor H89 (20 μM) could also block the stimulatory effects of NKB/NKBRP (1 μM) on SLα (Fig.4C) and PRL secretion and mRNA expression (Fig.4D). In parallel experiments, NKB- and NKBRP-induced SLα release and transcript expression in carp pituitary cells were abrogated by simultaneous incubation with the PLC inactivator U73122 (10 μM), PKC inhibitor GF109203X (20 μM), and IP_3 receptor blocker 2-APB (100 μM), respectively (Fig. 5A). Similar blockade was also observed for the PRL responses expect that PKC inactivation by GF109203X was not able to inhibit NKB- and NKBRP-induced PRL release and gene expression (Fig.5B).

In pituitary cells preloaded with the Ca^{2+}-sensitive dye Fura-2, NKB and NKBRP treatment (1 μM) consistently induced a rapid rise in fluorescence signals for intracellular Ca^{2+} ($[Ca^{2+}]i$) levels (Fig.6A). These Ca^{2+} responses were composed of an initial peak occurred within the first 30 sec followed by a shoulder phase with gradual reduction of the Ca^{2+} rise with levels maintained well above the basal. In parallel experiments, the shoulder phase but not peak phase could be abrogated by co-treatment with the voltage-sensitive Ca^{2+} channel (VSCC) blocker nifedipine (10 μM, Fig.6B) or removal of extra-cellular Ca^{2+} ($[Ca^{2+}]e$) using a Ca^{2+}-free culture medium (Fig.6C). Furthermore, the peak phase of the Ca^{2+} responses observed under the Ca^{2+}-free medium were markedly suppressed by the IP_3 receptor blocker 2-APB (100 μM, Fig.6D). In carp pituitary cells, SLα and PRL release and mRNA expression could be elevated dose-dependently by increasing levels of the Ca^{2+} ionphore A23187 (0.1-100 nM, Fig.6E). In contrast, NKB- and NKBRP-induced SLα (Fig.7A) and PRL secretion and gene expression (Fig.7B) were found to be attenuated/abolished by incubation with Ca^{2+}-free medium or co-treatment

with the VSCC inhibitor nifedipine (10 μM), CaM antagonist calmidazolium (1 μM) and CaMK-II blocker KN62 (5 μM), respectively. Parallel studies using Western blot also revealed that NKB and NKBRP were both effective in triggering rapid phosphorylation of the transcription factor CREB but with no effects on phosphorylation/total protein of other signaling kinases including $MEK_{1/2}$, $ERK_{1/2}$ and Akt (Supplemental Fig.4A-D). Of note, the stimulation on CREB phosphorylation could also be mimicked by parallel treatment with the AC activator forskolin (Supplemental Fig.4D).

Discussion

Although NKB is known to regulate LH release via modulation of kisspeptin/GnRH system in the hypothalamus (18, 19), little is known regarding its direct effects at the pituitary level. The comparative aspects of NKB become even more interesting with the discovery of the novel gene product NKBRP in zebrafish TAC3 (26, 27), the biological function of which is still at the early phase of investigation. To shed light on the pituitary actions of NKB and NKBRP in fish models, grass carp TAC3 was cloned and confirmed to be a single copy gene in the carp genome. Phylogenetic analysis reveals that the newly cloned TAC3 is a member of TAC3 subfamily closely related to zebrafish TAC3a. Although the NKBRP sequence could not be found in TAC3 of the bird and mammals, presumably due to a loss of segmentally duplicated gene fragment in TAC3 during tetrapod evolution (28), the a.a. sequences of NKB and NKBRP are highly conserved (if not identical) among fish species. Since the two dibasic cleavage sites (KR & GRR) for NKB were also found in the flanking regions of NKBRP in grass carp TAC3 and the GRR motif is well-documented as the processing site for peptidyl-glycine α-amidating monooxyenase (36), it would be expected that the mature peptide of NKBRP with α-amidation in the C-terminal can be released in a way similar to that of NKB. This idea is consistent with the common observations that the C-terminal α-amidation is essential for the bioactivity and receptor binding for tachykinins in mammals (37).

In grass carp, similar to zebrafish (27), TAC3 was found to be widely expressed at the tissue level,

with high levels in the brain, intestine and gonad, and to a lower extent in the liver, gills and muscle. Although TAC3 was not detected in the spleen, low level of TAC3 signals could still be noted in other tissues and brain areas including the pituitary. In our study, high levels of TAC3 expression in the brain and intestine are consistent with the functional role of tachykinins as neurotransmitters/neuro-modulators within the CNS (1) as well as a major component of gut/brain peptides regulating motility and secretory functions in gastrointestinal tract (38). In mammals (e.g., rat), TAC3 is widely expressed in various components of the reproductive system, including the placenta (39), uterus (40, 41), ovary (13), prostate gland and testis (42). In testis, TAC3 can be detected in Leydig cells and NKB together with SP and NKA are known to play a role in sperm motility (43). Although TAC3 expression in granulosa cells has been reported in the ovary (13), its role in folliculogenesis/oocyte maturation is still unclear. In grass carp, high level of TAC3 signal could be identified in the hypothalamus, which corroborates with the recent findings of NKB-containing neurons in the hypothalamus of zebrafish (26) and NKB modulation of hypothalamic kisspeptin/GnRH system in mammalian models (18, 19). Of note, NK1R, NK2R and NK3R expression could also be located in carp pituitary cells. Together with the detection of TAC3 signal in the carp pituitary, these findings raise the possibility that TAC3 gene products may act in an autocrine/paracrine manner to regulate pituitary functions in carp species.

In mammals, except for a single report with NKB induction of PRL release in rat pituitary cells (24), the studies on the pituitary actions of NKB are rather limited. Recently, attempt has been made using pituitary cell lines to test NKB actions. In rat GH_3 lactotrophs, NKB had no effects on basal but elevated TRH-induced PRL promoter activity, while similar treatment in LβT2 gonadotrophs did not alter basal as well as GnRH-induced LHβ and FSHβ promoter activities (25). In carp pituitary cells, we have the novel findings that the gene products of carp TAC3, NKB and NKBRP, could increase PRL and SLα release, cell content, total production and mRNA levels in a time- and dose-dependent manner. These effects appear to be specific for PRL and SLα, as the treatment did not affect transcript expression of other pituitary hormones or modify basal levels of LH, GH and SLβ secretion. Similar to PRL, SL is also a member of GH gene lineage with pleiotropic functions in fish models, including background adaption, reproduction, acid-base balance, lipid metabolism and immune responses (44).

Two isoforms of SL, SLα and SLβ, have been identified in the fish pituitary, e.g., in zebrafish (45) and grass carp (29), and suspected to have overlapping and yet distinct functions (46). In our study, lower nanomolar doses of the NK2R agonist GR64349, but not the NK1R agonist HK-1 or NK3R agonist senktide, could mimic NKB/NKBRP-induced PRL release and mRNA expression in carp pituitary cells. Similar induction on SLα secretion and gene expression, however, were mimicked only by the NK3R agonist senktide. Consistent with these findings, the stimulation on PRL and SLα release and transcript levels induced by the two TAC3 gene products could be abolished selectively by the NK2R antagonist GR159897 and NK3R antagonist SB222200 respectively, whereas co-treatment with other NKR antagonists were found to have no effects. Since (i) NK2R and NK3R expression were found to overlap respectively with PRL cells within the RPD and SLα cells located in NIL of the carp pituitary, and (ii) NK2R and NK3R were the only NKR subtypes detected separately in immuno-identified PRL cells and SLα cells isolated by LCM technique, it is likely that the two TAC3 gene products can act at the pituitary level to induce PRL and SLα synthesis and secretion by differential activation of NK2R and NK3R expressed in the respective cell types. Given that NKB and NKBRP did not modify LH release or LHβ mRNA levels in carp pituitary cells, our results do not support the pituitary action of TAC3 gene products on LH regulation in grass carp.

In mammals, NKR via G protein activation (G_o & $G_{q/11}$) or arrestin-dependent scaffolding following receptor internalization are known to trigger biological actions by coupling with a multitude of post-receptor signaling cascades (5-10), but similar information in lower vertebrates, including amphibians and fish, is still lacking. In carp pituitary cells, NKB and NKBRP could induce cAMP production in a dose-dependent manner while increasing the functional levels of cAMP with a membrane-permeant cAMP analog 8Br.cAMP or stimulating cAMP synthesis using the AC activator forskolin could mimic the stimulatory effects of the two TAC3 gene products on PRL and SLα release and mRNA levels. In agreement with these findings, NKB/NKBRP-induced PRL and SLα secretion and gene expression could be negated by AC inactivation with MDL12330A or PKA blockade with H89. Judging from the previous reports on cAMP production triggered by mammalian NK2R (9) and NK3R activation (8), it would be logical to conclude that the AC/cAMP/PKA pathway is involved in PRL and SLα synthesis

and secretion induced by the two TAC3 gene products, probably via differential activation of the two NKR subtypes expressed in the carp pituitary. Although NKB and NKBRP treatment did not affect $MEK_{1/2}$, $ERK_{1/2}$ and Akt phosphorylation in carp pituitary cells, rapid phosphorylation of CREB was noted and this stimulatory effect could be mimicked by increasing cAMP production with forskolin. Apparently, MAPK and PI3K/Akt pathways are not involved in the pituitary actions of the two TAC3 gene products. Our findings on CREB phosphorylation also raise the possibility that CREB activation may be working downstream of AC/cAMP/PKA cascades coupled to NK2R and NK3R to up-regulate PRL and SLα gene transcription, respectively.

Since IP_3 production and Ca^{2+} mobilization have been documented for mammalian NKR expressed in various cell types, e.g., NK1R in CHO cells (8), NK2R in HEK293 cells (9) and NK3R in HASM cells (10), the functional role of PLC- and Ca^{2+}-dependent cascades in the pituitary actions of NKB and NKBRP were also examined. In carp pituitary cells, PLC inhibition by U73122 and IP_3 receptor inactivation by 2-APB were both effective in blocking NKB/NKBRP-induced PRL and SLα secretion and transcript expression. Similar blockade on SLα release and gene expression were also observed with PKC inactivation by GF109203X, which is consistent with our previous demonstration of SLα mRNA expression in carp pituitary cells induced by the PKC activator TPA and diacylglyercol (DAG) analog DiC8 (47). The corresponding PRL responses in the same experiment, however, were found to be insensitive to PKC blockade. These results suggest that the PLC/IP_3/PKC cascade was involved in SLα secretion and gene expression induced by the two TAC3 gene products. Apparently, the same pathway was also a part of the post-receptor signaling mediating the corresponding PRL responses in the carp pituitary except that the PKC component was not involved. A similar finding with differential involvement of PKC in PACAP-induced SLα and SLβ expression via PLC-dependent mechanisms has been recently reported in the carp pituitary (47). Given that multiple isoforms of PKC have been identified in the fish pituitary, e.g., goldfish (48), and some of them, e.g., PKC_{ζ} and PKC_{η}, are known to have atypical pharmacological properties (49), we do not exclude the possibility that PKC isoforms insensitive to GF109203X might be involved in the PRL responses occurred in the carp pituitary.

In our study, Ca^{2+} imaging also revealed that NKB and NKBRP were both effective in triggering a biphasic Ca^{2+} rise with an initial peak followed by a shoulder phase in carp pituitary cells. The peak phase of the Ca^{2+} response was insensitive to removal of extracellular Ca^{2+} ($[Ca^{2+}]e$) using a Ca^{2+}-free medium but could be negated by IP_3 receptor inactivation with 2-APB, indicating that it was the result of $[Ca^{2+}]i$ mobilization in IP_3-sensitive Ca^{2+} stores. The shoulder phase, in contrast, was sensitive to $[Ca^{2+}]e$ removal and blocked by VSCC inhibition using nifedipine, suggesting that this delayed Ca^{2+} response was caused by $[Ca^{2+}]e$ entry via VSCC. In carp pituitary cells, Ca^{2+} rise triggered by VSCC activation using Bay K8644 is known to elevate GH (35) and SLα mRNA levels (47), suggesting that the Ca^{2+} signals are functionally coupled with pituitary hormone expression. Consistent with this idea, $[Ca^{2+}]e$ entry induced by the Ca^{2+} ionophore A23187 was found to up-regulate PRL and SLα secretion and transcript levels. Furthermore, NKB- and NKBRP-induced PRL and SLα release and mRNA expression could be inhibited by removing $[Ca^{2+}]e$ using Ca^{2+}-free medium, blockade of VSCC with nifedipine, antagonizing endogenous CaM by calmidazolium, or inactivating CaMK-II using KN62. These results, as a whole, suggest that the Ca^{2+} rise triggered by NKB and NKBRP via $[Ca^{2+}]e$ entry and $[Ca^{2+}]i$ mobilization could induce PRL and SLα secretion and gene expression in the respective cell types via the Ca^{2+}/CaM/CaMK-II cascade. In mammals, biphasic Ca^{2+} responses with initial peak dependent on IP_3 production and delayed shoulder phase dependent on $[Ca^{2+}]e$ entry via VSCC have been reported in rat pituitary cells after SP treatment (50). $[Ca^{2+}]i$ mobilization during the peak phase is consistent with the role of IP_3 receptors as the intracellular Ca^{2+} channels for $[Ca^{2+}]i$ release from IP_3-sensitive Ca^{2+} stores (51). In pituitary cell lines (e.g., GH_3 cells), PKA and PKC activation are also known to up-regulate VSCC activity (52), which may contribute to $[Ca^{2+}]e$ entry during the shoulder phase. To our knowledge, the biphasic Ca^{2+} response linked with NKB and the functional involvement of CaM and CaMK-II in the pituitary actions of tachykinins have not been reported in mammals.

In summary, we have cloned grass carp TAC3, characterized its gene copy number, and structurally confirmed the presence of the coding sequences of two mature peptides in its preprohormone, namely the fish version of NKB and a novel tachykinin-like peptide called NKBRP. In grass carp, TAC3 was found to be widely expressed in various tissues and brain areas, including the hypothalamo-pituitary

axis. At the pituitary level, the two TAC3 gene products, NKB and NKBRP, could both trigger PRL and SLα secretion, protein production and transcript expression, probably via differential activation of NK2R and NK3R expressed in PRL cells and SLα cells, respectively (Fig.8). NKB and NKBRP, however, did not have direct effects on LH regulation in the carp pituitary. Using a pharmacological approach, the AC/cAMP/PKA, PLC/IP_3/PKC and Ca^{2+}/CaM/CaMK-II cascades were shown to be involved in NKB- and NKBRP-induced SLα secretion and gene expression. The signal transduction for the corresponding PRL responses was also similar to that of SLα, except that the PKC component coupled to PLC activation was not involved. Our findings for the first time provide evidence that the TAC3 gene products in fish model, NKB and NKBRP, could stimulate PRL and SLα synthesis and secretion via direct actions at the pituitary level through activation of different NKR subtypes coupled to overlapping and yet distinct post-receptor signaling mechanisms.

(5182 words)

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Legends

Fig.1. Sequence analysis, genomic Southern and tissue distribution of grass carp TAC3. (A) Protein sequence alignment of grass carp TAC3 with that of other vertebrates using Clustal-W algorithm with MacVector program. The conserved a.a. residues are boxed in grey and the dibasic protein cleavage sites (KR & GRR) are marked with inverted triangles. (B) Phylogenetic analysis of TAC3 nucleotide sequences using the neighbor-joining method with MEGA 6.0. The numbers presented in the guide-tree are the percentage of bootstrap values based on 1000 bootstraps. Ciona TAC3, a representative of the invertebrate sequence, was used as an out-group. (C) Southern blot of carp TAC3. Genomic DNA was isolated from whole blood of grass carp, digested with restriction enzymes as indicated, resolved by agarose gel electrophoresis, and subjected to Southern blot by hybridization with a DIG-labeled cDNA probe for carp TAC3. (D) Tissue expression profile of carp TAC3. Total RNA was isolated from selected tissues and brain areas in grass carp and subjected to RT-PCR using primers specific for TAC3 (TGTCAGCAGTCAGAGTCTCAAAG & AACCCACGACGAAACCTCAGT). PCR reaction was fixed at 40 cycles with 30 sec at 94℃ for denaturing, 30 sec at 56 ℃ for annealing and 30 sec at 72 ℃ for extension. Authenticity of PCR products was confirmed by Southern blot using the DIG-labeled TAC3 probe and parallel RT-PCR for β-actin was used as the internal control.

Fig.2. Effects of TAC3 gene products on SLα and PRL synthesis and secretion in carp pituitary cells. Time course of grass carp NKB (1 μM) and NKBRP treatment (1 μM) on (A) SLα and PRL secretion, cell content and total production, and (B) SLα and PRL mRNA expression in carp pituitary cells. (C) Dose-dependence of 24-hr treatment with increasing levels of NKB and NKBRP (0.1-1000 nM) on SLα and PRL secretion and mRNA expression. After drug treatment, culture medium was harvested for measurement of hormone release and cell lysate was prepared for monitoring hormone content in pituitary cells. In parallel experiments, total RNA was isolated for real-time PCR of SLα and PRL mRNA using primers specific for the respective gene targets (ACCCACTGTACTTCAATCTCC & CGTCGTAACGATCAAGAGTAG for SLα and CTCAGCACCTCTCTCACCAATGACC & GCGG AAGCAGGACAACAGAAAATG for PRL). Real-time PCR was routinely performed for 35 cycles

with denaturation at 94 ℃ for 30 sec, annealing at 52 ℃ for SLα or 59 ℃ for PRL for 30 sec, and extension at 72 ℃ for 30 sec. In the data presented (Mean ± SEM), the groups denoted by different letters represent a significant difference at $P < 0.05$ (ANOVA followed by Dunnett's test).

Fig.3. Receptor specificity for SLα and PRL regulation by TAC3 gene products in carp pituitary cells. Effects of NKR antagonists on NKB- and NKBRP-induced (A) SLα and (B) PRL release and transcript expression. Pituitary cells were treated for 24 hr with NKB (100 nM) or NKBRP (100 nM) in the presence or absence of the NK1R antagonist L732138 (1 μM), NK2R antagonist GR159897 (1 μM) and NK3R antagonist SB222200 (1 μM), respectively. Effects of NKR agonists on SLα and PRL transcript expression (C) and hormone secretion (D). For SLα and PRL mRNA expression, pituitary cells were treated for 24 hr with increasing levels (01-1000 nM) of the NK1R agonist HK-1, NK2R agonist GR64349 and NK3R agonist senktide, respectively. For the experiments on hormone release, only a single dose at 10 nM was tested for 24 hr treatment for the three NKR agonists. (E) Cell-type specific expression of NK1R, NK2R and NK3R in carp pituitary cells. Pure populations of immuno-identified GH cells, PRL cells, SLα cells and SLβ cells (~250 cells/PCR sample) were isolated from grass carp pituitary cells using LCM technique and subjected to RT-PCR using primers specific for NK1R, NK2R and NK3R respectively (NK1R: GGAATGGATTCGCTCATCACTT & TAACGGTGT TGAATGCGGAC; NK2R: AGATGATGATAGTGGTGGTGAC & GCAGTAGAGATGGGGTTGTA; NK3R: GCCAAGAGAAAGGTTGTGAAGA & GTGTACATGCTGCTCTGGCG). PCR reactions were conducted for 50 cycles with 30 sec at 94 ℃ for denaturing, 30 sec at 54 ℃ for annealing and 30 sec at 72 ℃ for extension. In this study, RT-PCR of the three NKR subtypes in mixed populations of carp pituitary cells was used as a positive control while RT-PCR for β-actin was used as the internal control.

Fig.4. Functional role of cAMP-dependent pathway in pituitary regulation of SLα and PRL by TAC3 gene products. (A) Effects of 20-min incubation with increasing levels (0.1-1000 nM) of NKB and NKBRP on cAMP production in carp pituitary cells. (B) Effects of 24-hr treatment with the membrane-permeant cAMP analog 8Br.cAMP (10-1000 μM) or AC activator forskolin (1 μM, FSK) on SLα and

PRL mRNA expression. Effects of 24-hr co-treatment with the AC inhibitor MDL12330A (20 μM) or PKA inhibitor H89 (20 μM) on NKB (1 μM)- and NKBRP (1 μM)-induced (C) SLα and (D) PRL release and mRNA expression. After drug treatment, culture medium was harvested for measurement of hormone release. The remaining cells were either extracted for cAMP production or used for total RNA preparation for subsequent real-time PCR of the respective gene targets.

Fig.5. Functional role of PLC-dependent pathway in pituitary regulation of SLα and PRL by TAC3 gene products. Effects of 24-hr co-treatment with the PLC inhibitor U073122 (10 μM), PKC inhibitor GF109203X (20 μM) or IP_3 receptor blocker 2-APB (100 μM) on NKB (1 μM)- and NKBRP (1 μM)-induced (A) SLα and (B) PRL secretion and mRNA expression in carp pituitary cells. After drug treatment, culture medium was harvested for hormone release and total RNA was extracted from the remaining cells for real-time PCR of the respective gene targets.

Fig.6. Functional coupling of TAC3 gene products with Ca^{2+} signaling in carp pituitary cells. (A) Effects of NKB (1 μM) and NKBRP (1 μM) on intracellular Ca^{2+} levels in carp pituitary cells. Parallel treatment with the vehicle (Veh) used for dissolving the TAC3 gene products was used as the solvent control. Effects of (B) co-treatment with the VSCC blocker Nifedipine (10μM, Nifed) or (C) removal of extracellular Ca^{2+} using a Ca^{2+}-free medium on Ca^{2+} signals triggered by NKB (1 μM) and NKBRP (1 μM) in carp pituitary cells. (D) Effects of co-treatment with the IP_3 receptor blocker 2-APB (100 μM) on NKB (1 μM) and NKBRP (1 μM)-induced Ca^{2+} responses in pituitary cells incubated with the Ca^{2+}-free medium. (E) Effects of increasing doses of the Ca^{2+} ionophore A23187 (0.1 - 100 nM, 24 hr) on SLα and PRL release and mRNA expression in carp pituitary cells. In the experiments for Ca^{2+} measurement, pituitary cells were pre-loaded with the Ca^{2+}-sensitive dye Fura-2 and Ca^{2+} data were presented as a ratio of the fluorescence emission obtained with excitation at 340 nm and 380 nm, respectively (as "F340/F380 Ratio"). For the studies on SLα and PRL secretion and gene expression, culture medium was harvested after drug treatment for hormone release and total RNA was extracted from pituitary cells for real-time PCR of the respective gene targets.

Fig.7. Functional role of Ca^{2+}-dependent pathway in pituitary regulation of SLα and PRL by TAC3 gene products. Effects of 24-hr incubation with Ca^{2+}-free medium or co-treatment with the VSCC blocker Nifedipine (10 μM), CaM antagonist calmidazolium (1 μM) or CaMK-II inactivator KN62 (5 μM), respectively, on NKB (1 μM)- and NKBRP (1 μM)-induced (A) SLα and (B) PRL secretion and transcript expression in carp pituitary cells. After drug treatment, culture medium was harvested for hormone release and total RNA was extracted from the remaining cells for real-time PCR of the respective gene targets.

Fig.8. Working model of NKB and NKBRP induction of SLα and PRL synthesis and secretion in carp pituitary cells. In grass carp, two mature peptides, NKB and NKBRP, can be produced from TAC3 preprohormone, presumably by protein processing via the two dibasic cleavage sites (KR & GRR) flanking the respective gene products. These two TAC3 gene products through differential activation of NK2R expressed in PRL cells and NK3R expressed in SLα cells can up-regulate PRL and SLα transcript expression, protein production and hormone secretion in the respective cell types within the carp pituitary. These stimulatory effects, except for a lack of PKC involvement in the PRL responses, appear to be mediated by the AC/cAMP/PKA, PLC/IP_3/PKC and Ca^{2+}/CaM/CMK-II cascades.

Fig.1

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Fig.1

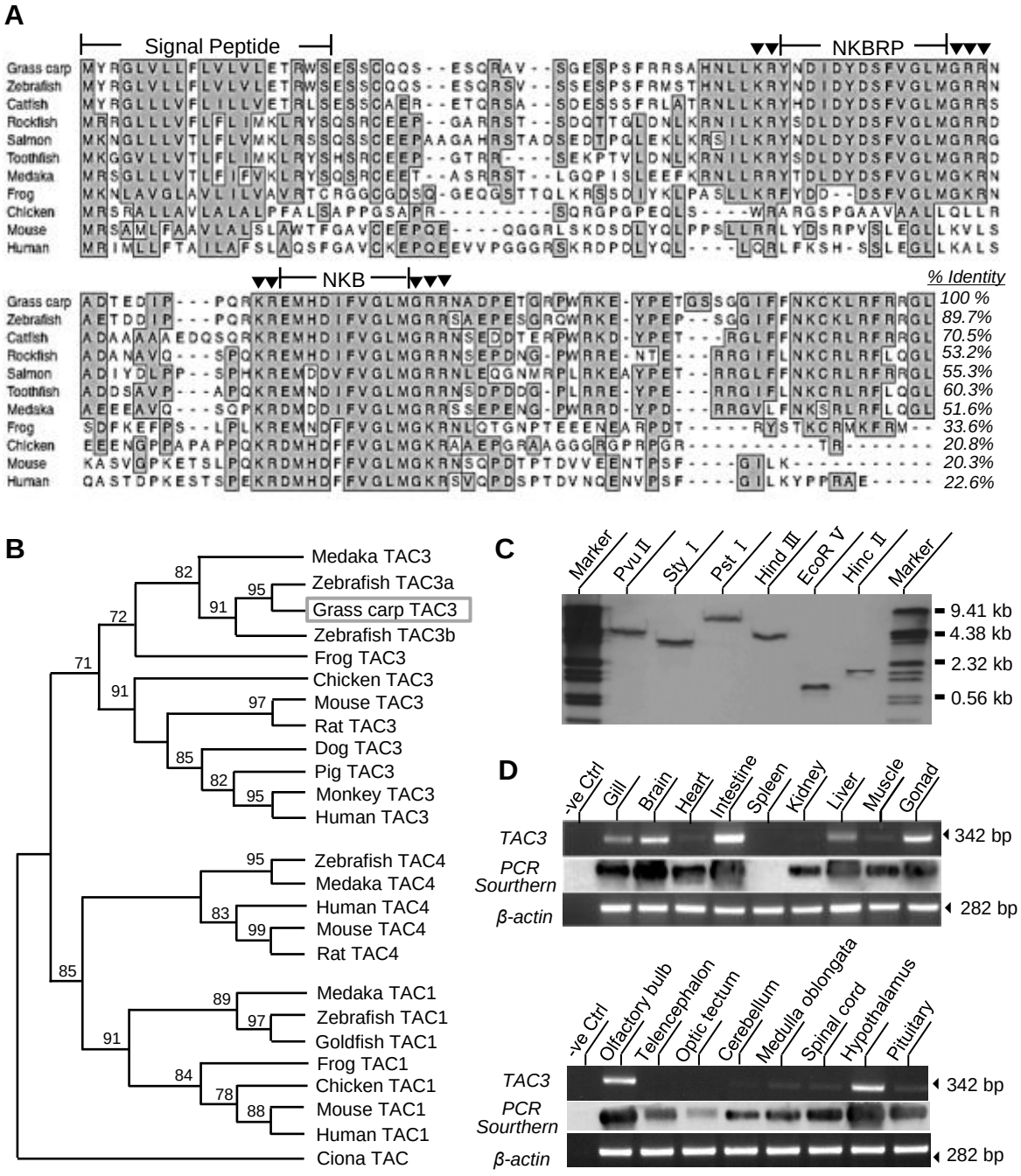

Fig.2

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Fig.2

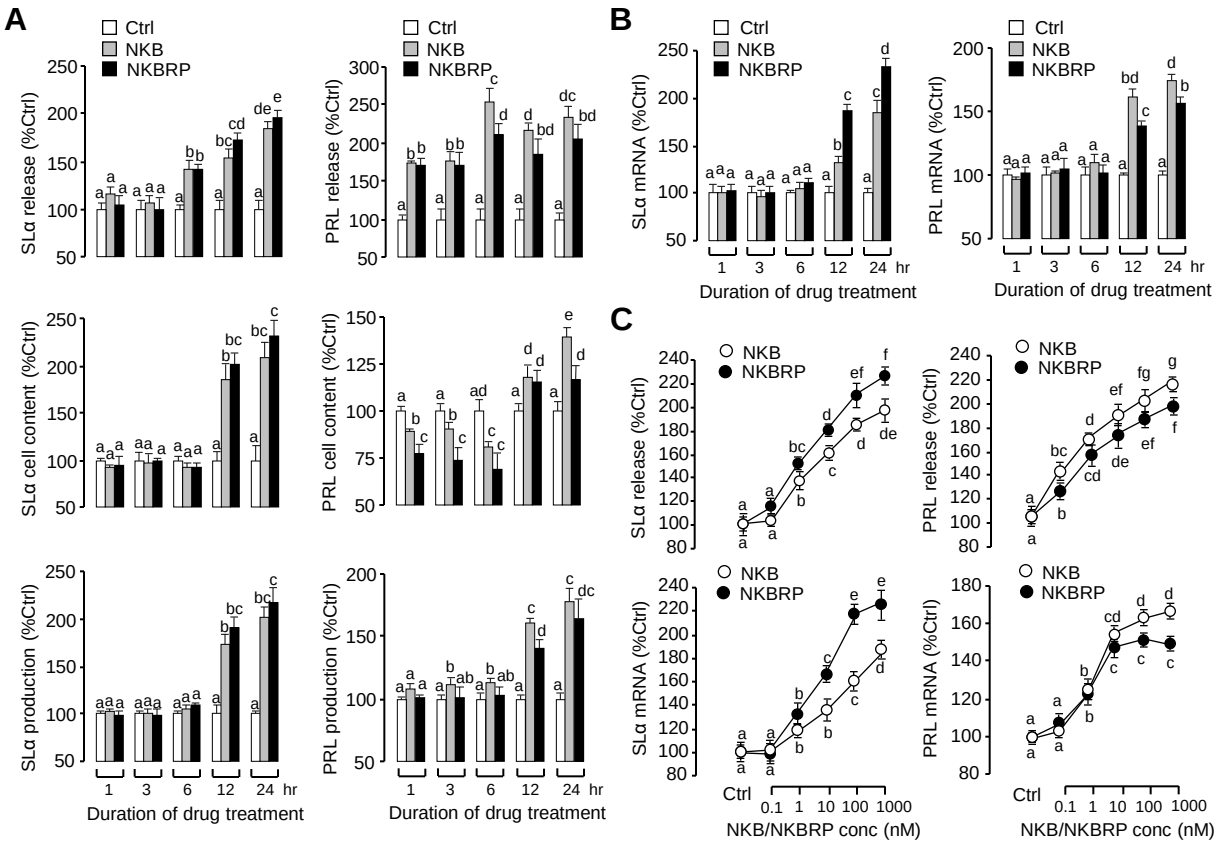

Fig.3

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Fig.3

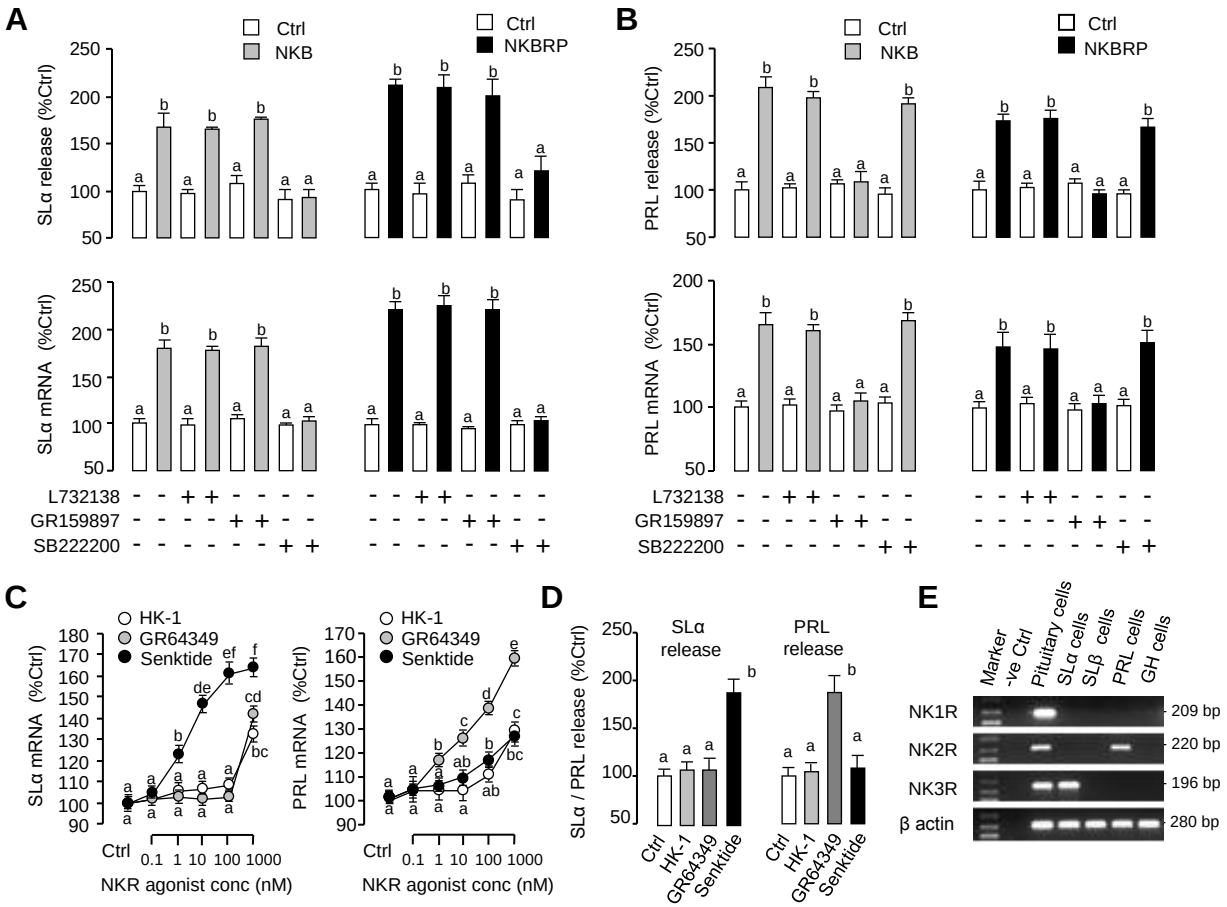

Fig.4
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Fig.4

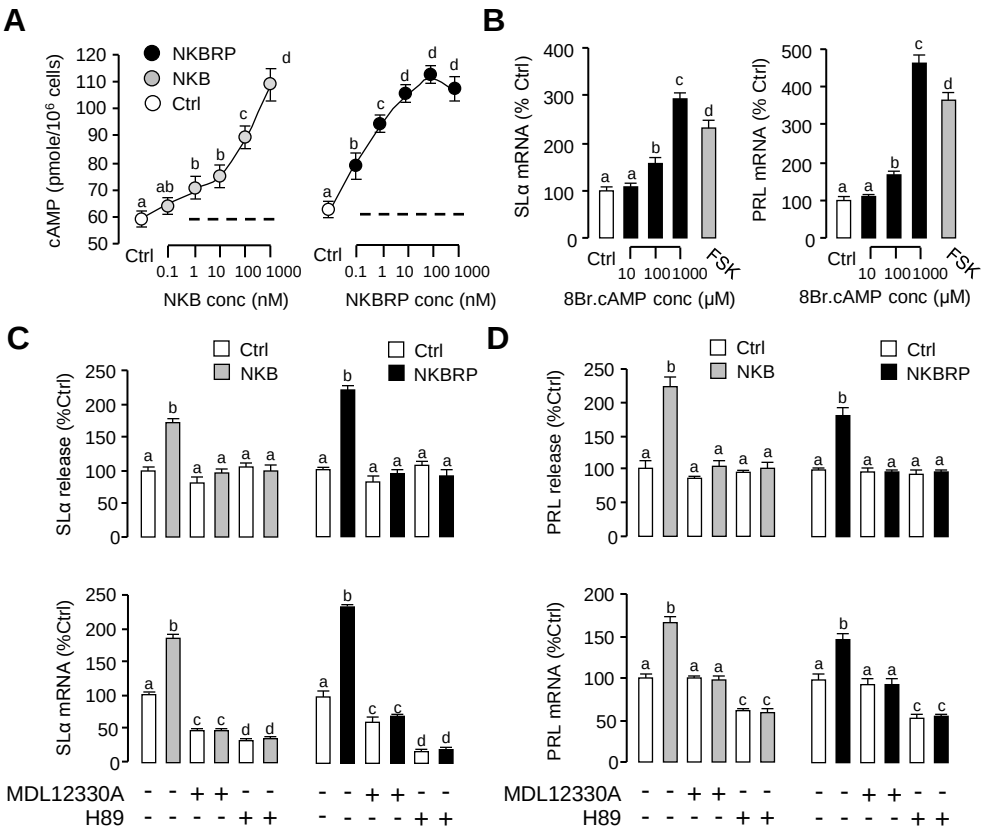

Fig.5
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Fig.5

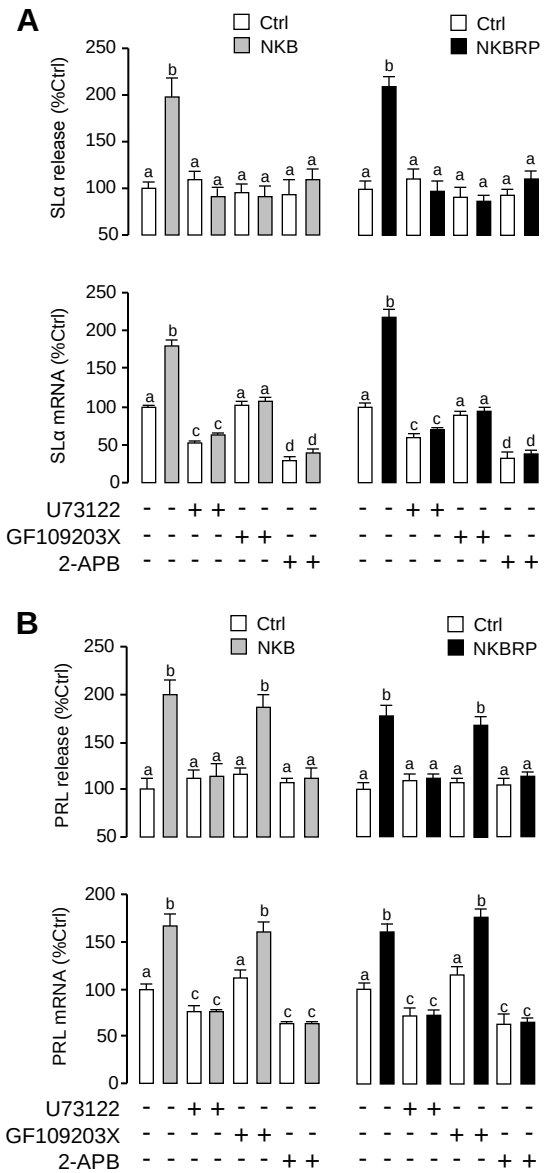

Fig.6
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Fig.6

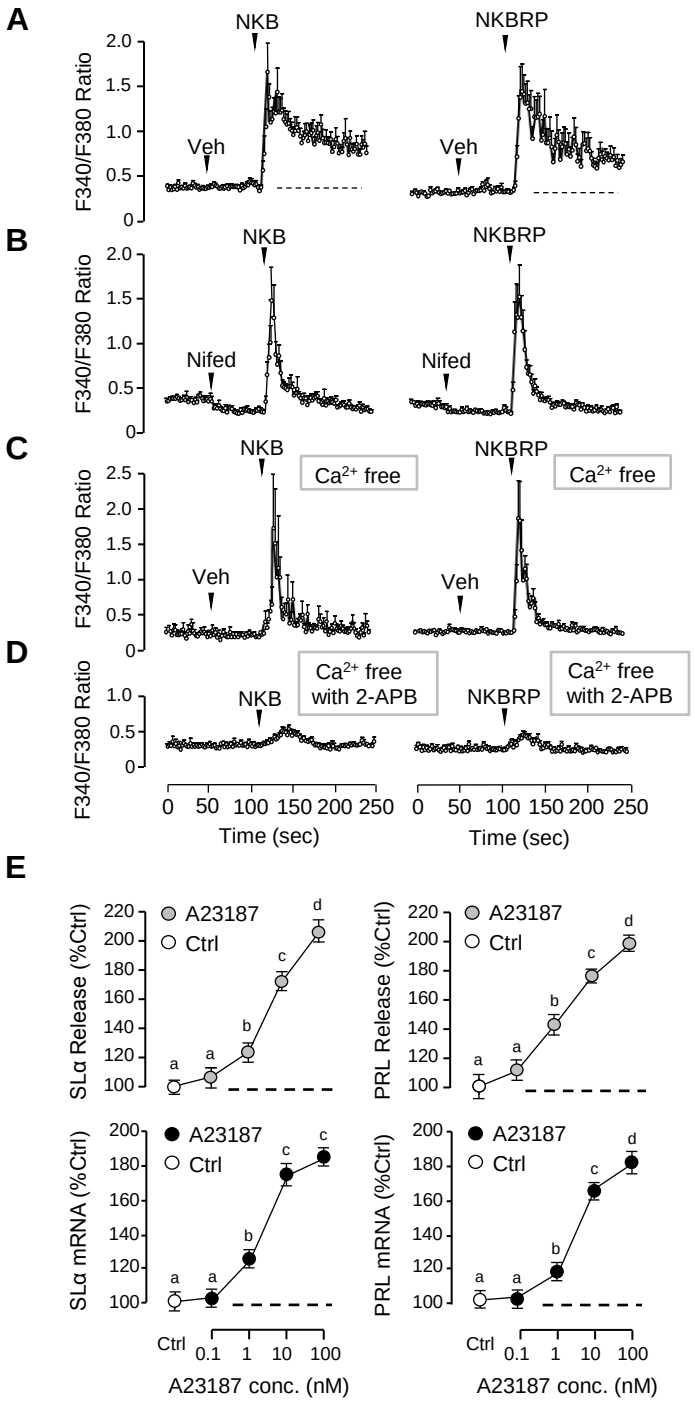

Fig.7

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Fig.7

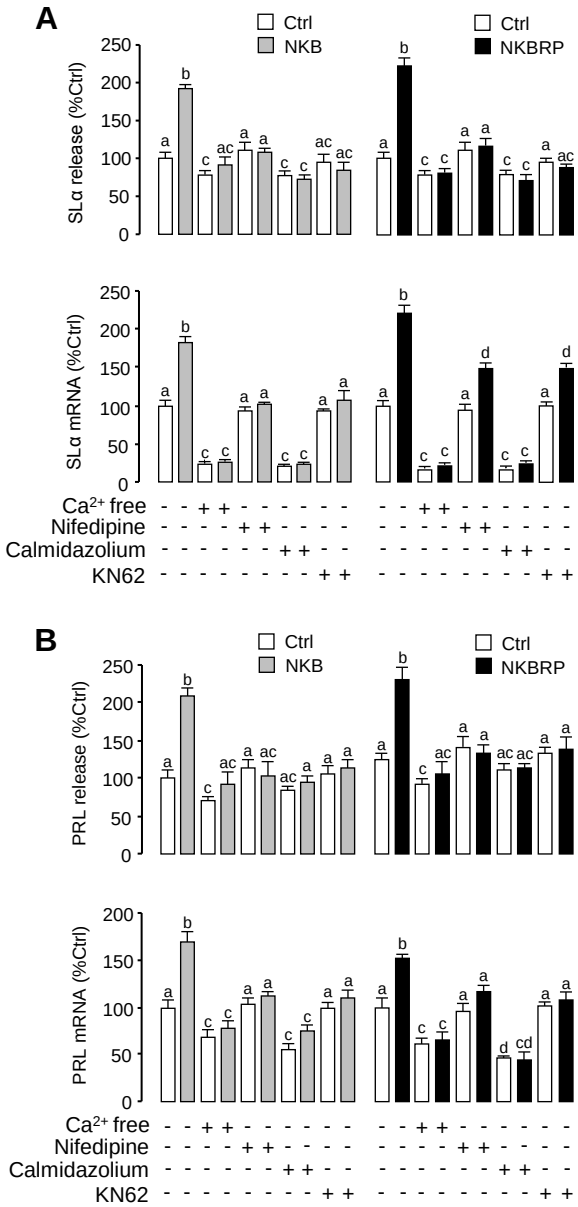

Fig.8
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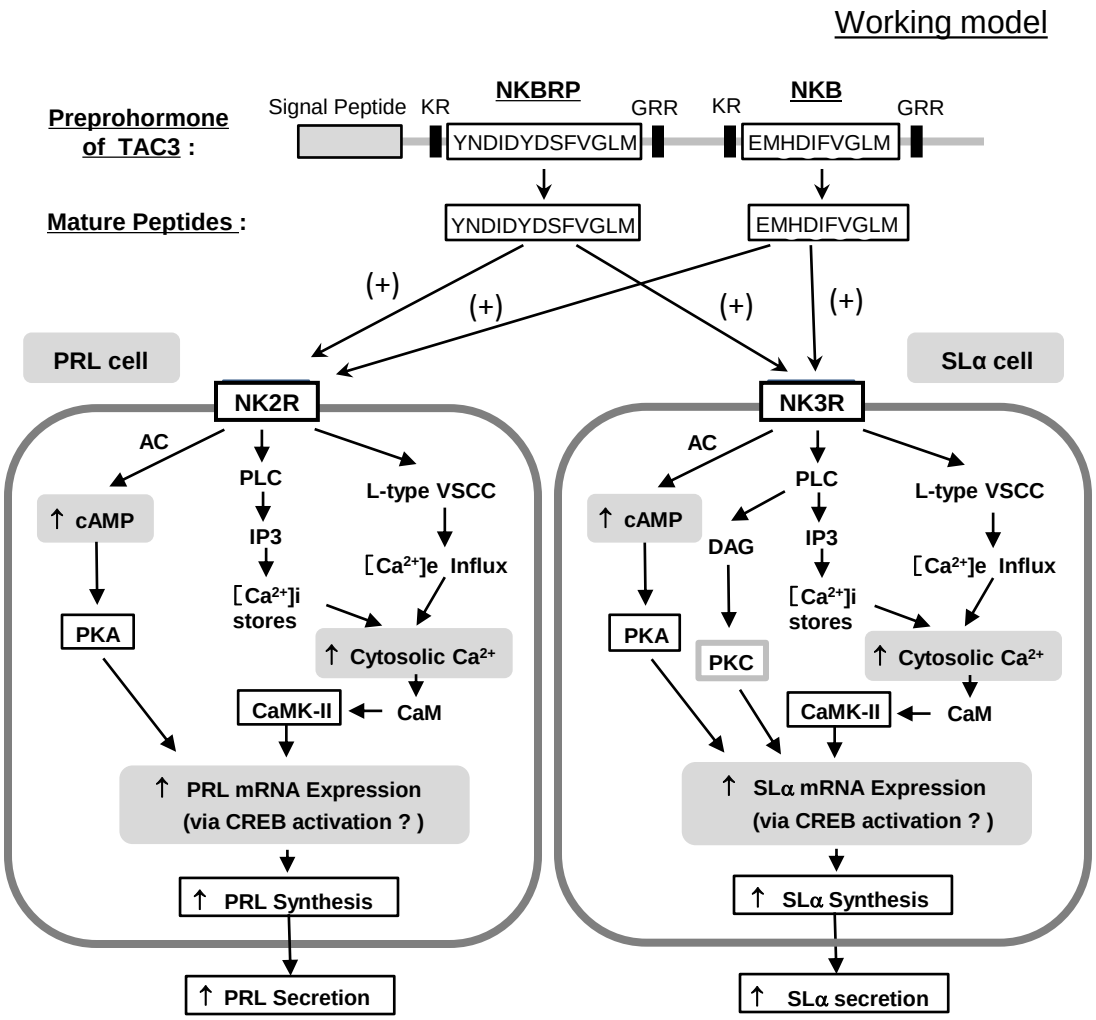
Fig.8



Supplemental Fig.1

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Supplemental Fig.2

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Supplemental Fig.3
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Supplemental Fig.4

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Antibody Table

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Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Phospho-MEK1/2 (Ser217/221)	a synthetic phosphopeptide (KLP-coupled) corresponding to residues around Ser217/221 of human MEK1/2.	Phospho-MEK1/2 (Ser217/221) mAb	Cell Signaling Technology, Inc., catalog #9154	monoclonal IgG in Rabbit	1:1,500 for WB
MEK1/2	a synthetic peptide (KLH coupled) covering the conserved region of human, rat and mouse MEK1/2.	MEK1/2 Antibody (for total MEK1/2)	Cell Signaling Technology, Inc., catalog #9122	polyclonal in Rabbit	1:1,500 for WB
Activated (Diphosphorylated) ERK1/2	a synthetic peptide (KLH coupled) with HTGFLTpEYpVAT sequence corresponding to the phosphorylated form of ERK-activation loop	Diphosphorylated ERK1/2 mAb	Sigma-Aldrich Co. , catalog #M8159	monoclonal IgG1 in Mouse	1:5,000 for WB
ERK-1/2	a synthetic peptide (KLH coupled) with RRITVEEALAHPYLEQ YYDPTDE sequence derived from subdomain-XI of human ERK1/2.	ERK1/2 Antibody (for total ERK1/2)	Sigma-Aldrich Co. , catalog #M5670	polyclonal in Rabbit	1:5,000 for WB
Phospho-Akt (Ser473)	a synthetic phosphopeptide (KLH-coupled) corresponding to residues surrounding Ser473 of mouse Akt.	Phospho-Akt (Ser473) Antibody	Cell Signaling Technology, Inc., catalog #9271	polyclonal in Rabbit	1:1,500 for WB
Akt	a synthetic peptide (KLH-coupled) derived from the carboxy-terminal sequence of mouse Akt.	Akt Antibody (for total Akt)	Cell Signaling Technology, Inc., catalog #9272	polyclonal in Rabbit	1:1,500 for WB
Phospho-CREB (Ser133)	a synthetic peptide (KLP-coupled) derived from the conserved region covering phosphorylated Ser133 of CREB.	Phospho-CREB (Ser133) Antibody	EMD Millipore，catalog #06-519	polyclonal in Rabbit	1:2,000 for WB