



Title	Novel pituitary actions of TAC3 gene products in fish model: Receptor specificity and signal transduction for prolactin and somatolactin regulation by neurokinin B (NKB) and NKB-related peptide in carp pituitary cells
Author(s)	Hu, G; He, M; Ko, WKW; Lin, C; Wong, AOL
Citation	Endocrinology, 2014, v. 155 n. 9, p. 3582-3596
Issued Date	2014
URL	http://hdl.handle.net/10722/204824
Rights	Creative Commons: Attribution 3.0 Hong Kong License

Endocrinology

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.

--Manuscript Draft--

Manuscript Number:	EN-14-1105R2
Full Title:	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.
Short Title:	Pituitary actions of TAC3 gene products
Article Type:	Original Article
Section/Category:	Neuroendocrinology
Corresponding Author:	Anderson O L Wong, Ph.D University of Hong Kong Hong Kong, China CHINA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Hong Kong
Corresponding Author's Secondary Institution:	
First Author:	Guangfu Hu, MSc
First Author Secondary Information:	
Order of Authors:	Guangfu Hu, MSc Mulan He, PhD Wendy K W Ko, BSc Chengyuan Lin, PhD Anderson O L Wong, Ph.D
Order of Authors Secondary Information:	
Abstract:	<p>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.</p>

1 (Revised version submitted to [Endocrinology](#) on May 15, 2014.)

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

5 Guangfu Hu, Mulan He, Wendy K. W. KO, Chengyuan Lin, and Anderson O. L. Wong

6 School of Biological Sciences, University of Hong Kong, Hong Kong, China.

7

8 Page Heading: Pituitary actions of TAC3 gene products

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

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

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

17

18 Funding Support: GRF Grants (to AOLW) from Research Grant Council (Hong Kong).

19

20 Disclosure: The authors have nothing to disclose for potential conflict of interest.

21

22

23 Please address correspondence and reprint request to:

24 Prof. Anderson O. L. Wong,

25 Endocrinology Division, School of Biological Sciences,

26 University of Hong Kong, Pokfulam Road, Hong Kong, China.

27 (Phone: 852-2299-0863, Fax: 852-2299-9114, Email: olwong@hku.hk)

28

29 **Abstract**

30

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

48

49 (249 words)

50

51 **Introduction**

52

53 Tachykinins including substance P (SP), neurokinin A (NKA), neurokinin B (NKB), hemokinin-1
54 (HK-1) and endokinins constitute the largest group of neuropeptides in mammals. They are widely
55 expressed at the tissue level, functionally involved in vasodilation, gut motility, nociception, immuno-
56 modulation and neuroendocrine regulation (1), and have been implicated in clinical cases of asthma,
57 chronic pain, inflammatory bowel syndrome, Alzheimer's disease, anxiety attack and depression (2).
58 Multiple genes for tachykinins, e.g., TAC1 coding for SP and NKA, TAC3 coding for NKB and TAC4
59 coding for HK-1/endokinins, have been identified (3) and believed to be the result of gene duplication
60 occurred during vertebrate evolution (4). The biological actions of tachykinins are mediated by three
61 major types of neurokinin receptors (NKR), namely NK1R, NK2R and NK3R (3), which are class I
62 G-protein coupled receptors functionally coupled with PLC/IP₃/PKC, MAPK, cAMP/PKA and Ca²⁺-
63 dependent cascades (5-10). Individual NKR subtypes are known to exhibit differential binding for
64 different tachykinins, with NK1R preferring SP, NK2R preferring NKA and NK3R preferring NKB
65 respectively (3). With potential applications in clinical treatment, structure-activity relationship for
66 ligand/receptor interaction and development of agonists/antagonists with NKR subtype selectivity
67 have been a major focus of tachykinin research, particularly for rational design of novel therapeutics
68 (11).

69

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

79 within the ARC with NKB-induced kisspeptin release via NK3R to trigger GnRH secretion in the
80 hypothalamus (19, 20). NKB activation of kisspeptin output to GnRH neurons, however, can be
81 suppressed by local release of dynorphin from KNDy neurons and this inhibition is mediated via κ -
82 type opioid receptor (21) and highly dependent on steroid background of the animal (19). Although
83 NKB is involved in LH regulation via kisspeptin/GnRH modulation in the hypothalamus, its pituitary
84 actions cannot be excluded as NKR expression (e.g., NK1R & NK2R) can be detected in the pituitary
85 (22, 23) and NKB-induced prolactin (PRL) release (24) and enhancement of TRH-induced PRL gene
86 transcription (25) have been reported in rat pituitary cells and lactotroph cell line, respectively. Of
87 note, NK3R has not been identified at the pituitary level in mammals and the post-receptor signaling
88 for the pituitary actions of NKB are still unknown.

89
90 NKB regulation of reproductive functions has been recently extended to fish models. In zebrafish,
91 NKB/NK3R system has been identified (26) and NKB treatment can also elevate plasma LH levels
92 (27). Interestingly, the TAC3 gene in fish species not only encodes NKB but also a novel tachykinin
93 called NKB-related peptide (NKBRP/neurokinin F) (26, 27). Similar to NKB, NKBRP was effective
94 in activating NK3R (28) and inducing LH release in zebrafish (27). However, neuroanatomical studies
95 in zebrafish also reveal that NKB and kisspeptin are expressed in separate neuronal populations in
96 brain areas relevant to reproduction (26), suggesting that the “KNDy” system in fish may be different
97 from that of mammals. In this study, the pituitary actions of NKB and the novel peptide NKBRP were
98 examined in grass carp, a commercial fish in Asian countries with high market value. Grass carp TAC3
99 was cloned and its tissue expression, especially in the brain-pituitary axis, was characterized. Using
100 primary culture of carp pituitary cells as a model, we have demonstrated for the first time that the
101 gene products of TAC3, namely NKB and NKBRP, did not alter LH release/gene expression at the
102 pituitary level but rather serve as novel regulators for PRL and somatolactin α (SL α) synthesis and
103 secretion via overlapping post-receptor signaling mechanisms coupled to pituitary NK2R and NK3R,
104 respectively.

105

106

107 **Materials and Methods**

108

109 *Animal and test substances*

110

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

121

122 *Cloning, copy number and tissue expression of carp TAC3*

123

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

134

135 *PRL and SL α secretion, cell content and mRNA expression*

136

137 Grass carp pituitary cells prepared by trypsin/DNase digestion method (31) were seeded in 24-well
138 plates at $\sim 2.5 \times 10^6$ cells/ml/well and incubated with test substances for the duration as indicated. After
139 that, culture medium was harvested for monitoring PRL and SL α release and cell lysate was prepared
140 from pituitary cells (32) for measurement of cell content for the respective hormones. PRL and SL α
141 levels in these samples were quantified using RIA for PRL (33) and ELISA for SL α (34) with antisera
142 raised against the respective hormones in carp species. Total production of PRL and SL α in individual
143 wells were deduced *pro rata* based on the protein data for cell content and secretion for the respective
144 hormones. In parallel experiments, total RNA was isolated from pituitary cells, reversely transcribed,
145 and subjected to quantitative PCR for grass carp PRL and SL α mRNA using a RotorGene-Q Real-time
146 PCR system (Qiagen, Vaoencia, CA) (see Fig.2 legend for primer sequences & PCR conditions). In
147 these PCR assays, serial dilutions of plasmid DNA with PRL or SL α ORF sequences were used as the
148 standards for data calibration and parallel real-time PCR for β -actin was also conducted as the internal
149 control. To examine the possible coupling of NKB/NKBRP with various signaling targets, the cell
150 lysate prepared was also subjected to Western blot using antibodies for the phosphorylated form and
151 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
152 (32, 47). (See antibody table submitted for the details.)

153

154 *In situ hybridization of NK2R and NK3R in carp pituitary sections*

155

156 In situ hybridization was performed in consecutive carp pituitary sections (5 μ m thick) prefixed in
157 4% paraformaldehyde as described previously (29) using DIG-labeled antisense riboprobes for carp
158 NK2R and NK3R, respectively. Parallel hybridization with the corresponding sense-strand riboprobes
159 was used as the negative control. In carp pituitary sections, zonal distribution of the major cell types
160 was revealed by in situ hybridization using double-strand DIG-labeled cDNA probes for carp PRL,
161 GH, LH β and SL α , respectively. In this case, hybridization without adding cDNA probes was used as
162 the control.

163

164 *RT-PCR for NK1R expression in immuno-identified pituitary cells*

165

166 Carp pituitary cells were spread evenly onto glass slides ($\sim 5 \times 10^4$ cells/0.5 ml/slide), fixed in Bouin's
167 fixative and subjected to immunostaining with antisera for carp PRL (1:100,000), GH (1:50,000), SL α
168 (1:100,000) and SL β (1:100,000), respectively, using a Vectastain ABC Kit (Vector Lab, Burlingame,
169 CA). After that, immuno-identified PRL cells, GH cells, SL α and SL β cells were isolated separately by
170 laser capture microdissection (LCM) using a PixCell-II Cell Isolation System (Arcturus, MountView,
171 CA) (29). Total RNA was extracted from individual cell types and reversely transcribed for PCR
172 detection of grass carp NK1R (GenBank no: JQ254914), NK2R (GenBank no: JN105350) and NK3R
173 (GenBank no: JN105350) using primers specific for the respective receptor subtypes (see Fig.3 legend
174 for primer sequences & PCR conditions). Parallel RT-PCR for β -actin was also performed to serve as
175 the internal control.

176

177 *cAMP production and Ca²⁺ measurement in carp pituitary cells*

178

179 Pituitary cells were cultured at $\sim 3 \times 10^6$ cells/2 ml/35 mm dish and challenged with NKB/NKBRP in
180 the presence of the phosphodiesterase inhibitor IBMX (0.1 mM). After treatment, cAMP production
181 was quantified using a BioTrak [¹²⁵I]cAMP RIA Kit (Amersham, Piscataway, NJ) (30). For single-
182 cell Ca²⁺ imaging, pituitary cells were seeded onto coverslip ($\sim 0.5 \times 10^6$ cells/ml/coverslip), pre-loaded
183 with the Ca²⁺-sensitive dye Fura-2/AM (5 μ M, Molecular Probes, Eugene, Oregon), and tested for Ca²⁺
184 responses with drug treatment using a PTI DeltaScan Epifluorescence System (Photon Technology
185 International, West Sussex, UK) (35). Ca²⁺ signals were expressed as a ratio of fluorescence emission
186 at 510 nm obtained with excitation at 340 and 380 nm, respectively (as "F340/F380 Ratio").

187

188 *Data transformation and statistics*

189

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

191 ED₅₀ values of 8-15 ng/ml (for PRL) and 60-80 ng/ml (for SL α) were used for data calibration with
192 four-parameter logistic regression model of Prism 6.0 (GraphPad, San Diego, CA). For real-time PCR
193 of PRL and SL α mRNA, standard curves with dynamic range of 10⁵ and correlation coefficient ≥ 0.95
194 were used for data calibration with RotorGene-Q software 1.7 (Qiagen). Since no significant changes
195 were noted for β -actin mRNA in our studies, PRL and SL α mRNA data as well as the corresponding
196 protein data were simply transformed as a percentage of the mean value in the control group without
197 drug treatment (as “%Ctrl”). The data presented (as Mean \pm SEM) were pooled results from 6-8
198 experiments and analyzed with ANOVA followed by Dunnett’s test using Prism 6.0 and differences
199 between groups were considered as significant at P<0.05.

200

201

202 **Results**

203

204 *Cloning and sequence analysis of grass carp TAC3*

205

206 Using 5’/3’RACE, a full-length grass carp TAC3 cDNA (GenBank no: JN105351) was cloned and
207 found to be 631 bp in size with a 91 bp 5’UTR, 378 bp ORF encoding a 126 a.a. TAC3 precursor, and
208 173 bp 3’UTR with two putative polyadenylation signals ([Supplemental Fig.1](#)). Although the deduced
209 a.a. sequence of carp TAC3 precursor is only 20-23% homologous to that of mammalian counterparts,
210 the regions for signal peptide and NKB mature peptide are highly conserved among vertebrate species
211 ([Fig.1A](#)). Similar to other fish models, the a.a. sequence of NKBRP flanked by two dibasic cleavage
212 sites (KR & GRR) similar to that of NKB and with a tachykinin signature motif “FXGLM” in its C-
213 terminal can also be identified in the carp TAC3 precursor. Phylogenetic analysis based on nucleotide
214 sequences further confirms that the newly cloned cDNA can be clustered in the clade of fish TAC3
215 and is closely related to TAC3a reported in zebrafish ([Fig.1B](#)).

216

217 *Copy number and tissue expression of TAC3 gene*

218

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

228

229 *Pituitary hormone regulation by NKB and NKBRP*

230

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

244

245 *Receptor specificity for SL α and PRL regulation by TAC3 gene products*

246

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

262

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

275

276 *Signal transduction for SL α and PRL regulation by TAC3 gene products*

277

278 As shown in Fig.4A, cAMP production in carp pituitary cells could be elevated dose-dependently by
279 20-min treatment with NKB and NKBRP, respectively. Besides, 24-hr incubation with the membrane-
280 permeant cAMP analog 8Br.cAMP (10-1000 μ M) and adenylate cyclase (AC) activator forskolin (1
281 μ M) were both effective in up-regulating SL α and PRL mRNA levels (Fig.4B). Consistent with these
282 findings, co-treatment with the AC inhibitor MDL12330A (20 μ M) or PKA inhibitor H89 (20 μ M)
283 could also block the stimulatory effects of NKB/NKBRP (1 μ M) on SL α (Fig.4C) and PRL secretion
284 and mRNA expression (Fig.4D). In parallel experiments, NKB- and NKBRP-induced SL α release
285 and transcript expression in carp pituitary cells were abrogated by simultaneous incubation with the
286 PLC inactivator U73122 (10 μ M), PKC inhibitor GF109203X (20 μ M), and IP₃ receptor blocker 2-
287 APB (100 μ M), respectively (Fig. 5A). Similar blockade was also observed for the PRL responses
288 expect that PKC inactivation by GF109203X was not able to inhibit NKB- and NKBRP-induced PRL
289 release and gene expression (Fig.5B).

290

291 In pituitary cells preloaded with the Ca²⁺-sensitive dye Fura-2, NKB and NKBRP treatment (1 μ M)
292 consistently induced a rapid rise in fluorescence signals for intracellular Ca²⁺ ([Ca²⁺]_i) levels (Fig.6A).
293 These Ca²⁺ responses were composed of an initial peak occurred within the first 30 sec followed by a
294 shoulder phase with gradual reduction of the Ca²⁺ rise with levels maintained well above the basal. In
295 parallel experiments, the shoulder phase but not peak phase could be abrogated by co-treatment with
296 the voltage-sensitive Ca²⁺ channel (VSCC) blocker nifedipine (10 μ M, Fig.6B) or removal of extra-
297 cellular Ca²⁺ ([Ca²⁺]_e) using a Ca²⁺-free culture medium (Fig.6C). Furthermore, the peak phase of the
298 Ca²⁺ responses observed under the Ca²⁺-free medium were markedly suppressed by the IP₃ receptor
299 blocker 2-APB (100 μ M, Fig.6D). In carp pituitary cells, SL α and PRL release and mRNA expression
300 could be elevated dose-dependently by increasing levels of the Ca²⁺ ionophore A23187 (0.1-100 nM,
301 Fig.6E). In contrast, NKB- and NKBRP-induced SL α (Fig.7A) and PRL secretion and gene expression
302 (Fig.7B) were found to be attenuated/abolished by incubation with Ca²⁺-free medium or co-treatment

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

309

310

311 Discussion

312

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

329

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

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

347

348 In mammals, except for a single report with NKB induction of PRL release in rat pituitary cells (24),
349 the studies on the pituitary actions of NKB are rather limited. Recently, attempt has been made using
350 pituitary cell lines to test NKB actions. In rat GH₃ lactotrophs, NKB had no effects on basal but
351 elevated TRH-induced PRL promoter activity, while similar treatment in L β T2 gonadotrophs did not
352 alter basal as well as GnRH-induced LH β and FSH β promoter activities (25). In carp pituitary cells,
353 we have the novel findings that the gene products of carp TAC3, NKB and NKBRP, could increase
354 PRL and SL α release, cell content, total production and mRNA levels in a time- and dose-dependent
355 manner. These effects appear to be specific for PRL and SL α , as the treatment did not affect transcript
356 expression of other pituitary hormones or modify basal levels of LH, GH and SL β secretion. Similar
357 to PRL, SL is also a member of GH gene lineage with pleiotropic functions in fish models, including
358 background adaption, reproduction, acid-base balance, lipid metabolism and immune responses (44).

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

375

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

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

395

396 Since IP₃ production and Ca²⁺ mobilization have been documented for mammalian NKR expressed
397 in various cell types, e.g., NK1R in CHO cells (8), NK2R in HEK293 cells (9) and NK3R in HASM
398 cells (10), the functional role of PLC- and Ca²⁺-dependent cascades in the pituitary actions of NKB
399 and NKBRP were also examined. In carp pituitary cells, PLC inhibition by U73122 and IP₃ receptor
400 inactivation by 2-APB were both effective in blocking NKB/NKBRP-induced PRL and SL α secretion
401 and transcript expression. Similar blockade on SL α release and gene expression were also observed
402 with PKC inactivation by GF109203X, which is consistent with our previous demonstration of SL α
403 mRNA expression in carp pituitary cells induced by the PKC activator TPA and diacylglycerol (DAG)
404 analog DiC8 (47). The corresponding PRL responses in the same experiment, however, were found to
405 be insensitive to PKC blockade. These results suggest that the PLC/IP₃/PKC cascade was involved in
406 SL α secretion and gene expression induced by the two TAC3 gene products. Apparently, the same
407 pathway was also a part of the post-receptor signaling mediating the corresponding PRL responses in
408 the carp pituitary except that the PKC component was not involved. A similar finding with differential
409 involvement of PKC in PACAP-induced SL α and SL β expression via PLC-dependent mechanisms has
410 been recently reported in the carp pituitary (47). Given that multiple isoforms of PKC have been
411 identified in the fish pituitary, e.g., goldfish (48), and some of them, e.g., PKC ζ and PKC η , are known
412 to have atypical pharmacological properties (49), we do not exclude the possibility that PKC isoforms
413 insensitive to GF109203X might be involved in the PRL responses occurred in the carp pituitary.

414

415 In our study, Ca^{2+} imaging also revealed that NKB and NKBRP were both effective in triggering a
416 biphasic Ca^{2+} rise with an initial peak followed by a shoulder phase in carp pituitary cells. The peak
417 phase of the Ca^{2+} response was insensitive to removal of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_e$) using a Ca^{2+} -free
418 medium but could be negated by IP_3 receptor inactivation with 2-APB, indicating that it was the result
419 of $[\text{Ca}^{2+}]_i$ mobilization in IP_3 -sensitive Ca^{2+} stores. The shoulder phase, in contrast, was sensitive to
420 $[\text{Ca}^{2+}]_e$ removal and blocked by VSCC inhibition using nifedipine, suggesting that this delayed Ca^{2+}
421 response was caused by $[\text{Ca}^{2+}]_e$ entry via VSCC. In carp pituitary cells, Ca^{2+} rise triggered by VSCC
422 activation using Bay K8644 is known to elevate GH (35) and $\text{SL}\alpha$ mRNA levels (47), suggesting that
423 the Ca^{2+} signals are functionally coupled with pituitary hormone expression. Consistent with this idea,
424 $[\text{Ca}^{2+}]_e$ entry induced by the Ca^{2+} ionophore A23187 was found to up-regulate PRL and $\text{SL}\alpha$ secretion
425 and transcript levels. Furthermore, NKB- and NKBRP-induced PRL and $\text{SL}\alpha$ release and mRNA
426 expression could be inhibited by removing $[\text{Ca}^{2+}]_e$ using Ca^{2+} -free medium, blockade of VSCC with
427 nifedipine, antagonizing endogenous CaM by calmidazolium, or inactivating CaMK-II using KN62.
428 These results, as a whole, suggest that the Ca^{2+} rise triggered by NKB and NKBRP via $[\text{Ca}^{2+}]_e$ entry
429 and $[\text{Ca}^{2+}]_i$ mobilization could induce PRL and $\text{SL}\alpha$ secretion and gene expression in the respective
430 cell types via the Ca^{2+} /CaM/CaMK-II cascade. In mammals, biphasic Ca^{2+} responses with initial peak
431 dependent on IP_3 production and delayed shoulder phase dependent on $[\text{Ca}^{2+}]_e$ entry via VSCC have
432 been reported in rat pituitary cells after SP treatment (50). $[\text{Ca}^{2+}]_i$ mobilization during the peak phase
433 is consistent with the role of IP_3 receptors as the intracellular Ca^{2+} channels for $[\text{Ca}^{2+}]_i$ release from
434 IP_3 -sensitive Ca^{2+} stores (51). In pituitary cell lines (e.g., GH_3 cells), PKA and PKC activation are also
435 known to up-regulate VSCC activity (52), which may contribute to $[\text{Ca}^{2+}]_e$ entry during the shoulder
436 phase. To our knowledge, the biphasic Ca^{2+} response linked with NKB and the functional involvement
437 of CaM and CaMK-II in the pituitary actions of tachykinins have not been reported in mammals.

438

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

443 axis. At the pituitary level, the two TAC3 gene products, NKB and NKBRP, could both trigger PRL
444 and SL α secretion, protein production and transcript expression, probably via differential activation of
445 NK2R and NK3R expressed in PRL cells and SL α cells, respectively (Fig.8). NKB and NKBRP,
446 however, did not have direct effects on LH regulation in the carp pituitary. Using a pharmacological
447 approach, the AC/cAMP/PKA, PLC/IP₃/PKC and Ca²⁺/CaM/CaMK-II cascades were shown to be
448 involved in NKB- and NKBRP-induced SL α secretion and gene expression. The signal transduction
449 for the corresponding PRL responses was also similar to that of SL α , except that the PKC component
450 coupled to PLC activation was not involved. Our findings for the first time provide evidence that the
451 TAC3 gene products in fish model, NKB and NKBRP, could stimulate PRL and SL α synthesis and
452 secretion via direct actions at the pituitary level through activation of different NKR subtypes coupled
453 to overlapping and yet distinct post-receptor signaling mechanisms.

454

455 (5182 words)

456

457

458

459 **Acknowledgements**

460

461 The project was supported by GRF grants (to AOLW) from Research Grant Council (Hong Kong).
462 Financial support from the School of Biological Sciences (University of Hong Kong) in the form of a
463 postgraduate studentship (to GH) is also acknowledged. We also thank Profs Haoran Lin and Yong
464 Zhang (Sun Yatsen University, China) for sending us the zebrafish NKB and NKBRP for our initial
465 studies.

466

467

468 **References**

469

- 470 1. **Satake H, Kawada T** 2006 Overview of the primary structure, tissue-distribution, and functions of
471 tachykinins and their receptors. *Current drug targets* 7:963-974
- 472 2. **Lecci A, Maggi CA** 2003 Peripheral tachykinin receptors as potential therapeutic targets in visceral
473 diseases. *Expert Opin Ther Tar* 7:343-362
- 474 3. **Satake H, Aoyama M, Sekiguchi T, Kawada T** 2013 Insight into molecular and functional
475 diversity of tachykinins and their receptors. *Protein and Pept Lett* 20:615-627
- 476 4. **Conlon JM, Larhammar D** 2005 The evolution of neuroendocrine peptides. *Gen Comp*
477 *Endocrinol* 142:53-59
- 478 5. **Alblas J, van Etten I, Moolenaar WH** 1996 Truncated, desensitization-defective neurokinin
479 receptors mediate sustained MAP kinase activation, cell growth and transformation by a Ras-
480 independent mechanism. *EMBO J* 15:3351-3360
- 481 6. **DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O, Bunnett NW** 2000 The
482 proliferative and antiapoptotic effects of substance P are facilitated by formation of a β -arrestin-
483 dependent scaffolding complex. *Proc Natl Acad Sci U S A* 97:11086-11091
- 484 7. **Khawaja AM, Rogers DF** 1996 Tachykinins: Receptor to effector. *Int J Biochem Cell Biol* 28:721-
485 738
- 486 8. **Nakajima Y, Tsuchida K, Negishi M, Ito S, Nakanishi S** 1992 Direct linkage of three tachykinin
487 receptors to stimulation of both phosphatidylinositol hydrolysis and cyclic AMP cascades in
488 transfected Chinese hamster ovary cells. *J Biol Chem* 267:2437-2442
- 489 9. **Palanche T, Ilien B, Zoffmann S, Reck MP, Bucher B, Edelstein SJ, Galzi JL** 2001 The
490 neurokinin A receptor activates calcium and cAMP responses through distinct conformational states.
491 *J Biol Chem* 276: 34853-34861
- 492 10. **Mizuta K, Gallos G, Zhu DF, Mizuta F, Goubaeva F, Xu DB, Panettieri RA, Yang J, Emala**
493 **CW** 2008 Expression and coupling of neurokinin receptor subtypes to inositol phosphate and
494 calcium signaling pathways in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol*
495 *Physiol* 294:L523-L534

- 496 11. **Ganjiwale A, Cowsik SM** 2014 Molecular recognition of tachykinin receptor selective agonists:
497 Insights from structural studies. *Mini Rev Med Chem* [Epub ahead of print]
- 498 12. **Lehman MN, Coolen LM, Goodman RL** 2010 Kisspeptin/Neurokinin B/Dynorphin (KNDy) cells
499 of the arcuate nucleus: A central node in the control of gonadotropin-releasing hormone secretion.
500 *Endocrinology* 151:3479-3489
- 501 13. **Lasaga M, Debeljuk L** 2011 Tachykinins and the hypothalamo-pituitary-gonadal axis: An update.
502 *Peptides* 32:1972-1978
- 503 14. **Topaloglu AK** 2010 Neurokinin B signaling in puberty: Human and animal studies. *Mol Cell*
504 *Endocrinol* 324:64-69
- 505 15. **Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO,**
506 **Cook JR, Ozbek MN, Imamoglu S, Akalin NS, Yuksel B, O'Rahilly S, Semple RK** 2009 TAC3
507 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for neurokinin
508 B in the central control of reproduction. *Nat Genet* 41:354-358
- 509 16. **Guran T, Tolhurst G, Bereket A, Rocha N, Porter K, Turan S, Gribble FM, Kotan LD, Akcay**
510 **T, Atay Z, Canan H, Serin A, O'Rahilly S, Reimann F, Semple RK, Topaloglu AK** 2009
511 Hypogonadotropic hypogonadism due to a novel missense mutation in the first extracellular loop of
512 the neurokinin B receptor. *J Clin Endocr Metab* 94:3633-3639
- 513 17. **Navarro VM, Castellano JM, McConkey SM, Pineda R, Ruiz-Pino F, Pinilla L, Clifton DK,**
514 **Tena-Sempere M, Steiner RA** 2011 Interactions between kisspeptin and neurokinin B in the
515 control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab* 300:E202-E210
- 516 18. **Goodman RL, Hileman SM, Nestor CC, Porter KL, Connors JM, Hardy SL, Millar RP,**
517 **Cernea M, Coolen LM, Lehman MN** 2013 Kisspeptin, neurokinin B, and dynorphin act in the
518 arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology* 154:4259-
519 4269
- 520 19. **Ruka KA, Burger LL, Moenter SM** 2013 Regulation of arcuate neurons coexpressing kisspeptin,
521 neurokinin B, and dynorphin by modulators of neurokinin 3 and κ -opioid receptors in adult male
522 mice. *Endocrinology* 154:2761-2771
- 523 20. **Grachev P, Li XF, Lin YS, Hu MH, Elsamani L, Paterson SJ, Millar RP, Lightman SL,**

- 524 **O'Byrne KT** 2012 GPR54-dependent stimulation of luteinizing hormone secretion by neurokinin B
525 in prepubertal rats. PloS one 7:e44344
- 526 21. **Grachev P, Li XF, Kinsey-Jones JS, di Domenico AL, Millar RP, Lightman SL, O'Byrne KT**
527 2012 Suppression of the GnRH pulse generator by neurokinin B involves a κ -opioid receptor
528 dependent mechanism. Endocrinology 153:4894-4904
- 529 22. **Pisera D, Candolfi M, De Laurentiis A, Sellicovich A** 2003 Characterization of tachykinin NK2
530 receptor in the anterior pituitary gland. Life Sci 73:2421-2432
- 531 23. **Larsen PJ, Saermark T, Mau SE** 1992 Binding of an iodinated substance P analogue to cultured
532 anterior pituitary prolactin- and luteinizing hormone-containing cells. J Histochem Cytochem 40:
533 487-493
- 534 24. **Henriksen JS, Saermark T, Vilhardt H, Mau SE** 1995 Tachykinins induce secretion of prolactin
535 from perfused rat anterior pituitary cells by interactions with two different binding sites. Recept
536 Signal Transduct Res 15:529-541
- 537 25. **Mijiddorj T, Kanasaki H, Purwana IN, Oride A, Sukhbaatar U, Miyazaki K** 2012 Role of
538 neurokinin B and dynorphin A in pituitary gonadotroph and somatolactotroph cell lines. Endocr J
539 59:631-640
- 540 26. **Ogawa S, Ramadasan PN, Goschorska M, Anantharajah A, Ng KW, Parhar IS** 2012 Cloning
541 and expression of tachykinins and their association with kisspeptins in the brains of zebrafish. J
542 Comp Neurol 520:2991-3012
- 543 27. **Biran J, Palevitch O, Ben-Dor S, Levavi-Sivan B** 2012 Neurokinin Bs and neurokinin B receptors
544 in zebrafish: - Potential role in controlling fish reproduction. Proc Natl Acad Sci U S A 109:10269-
545 10274
- 546 28. **Zhou W, Li S, Liu Y, Qi X, Chen H, Cheng CH, Liu X, Zhang Y, Lin H** 2012 The evolution of
547 tachykinin/tachykinin receptor (TAC/TACR) in vertebrates and molecular identification of the
548 TAC3/TACR3 system in zebrafish (*Danio rerio*). Mol Cell Endocrinol 361:202-212
- 549 29. **Jiang Q, Ko WKW, Lerner EA, Chan KM, Wong AOL** 2008 Grass carp somatolactin: I.
550 Evidence for PACAP induction of somatolactin α and β gene expression via activation of pituitary
551 PAC-I receptors. Am J Physiol Endocrinol Metab 295:E463-E476

- 552 30. **Sze KH, Zhou H, Yang YH, He ML, Jiang YH, Wong AOL** 2007 Pituitary adenylate cyclase-
553 activating polypeptide (PACAP) as a growth hormone (GH)-releasing factor in grass carp: II.
554 Solution structure of a brain-specific PACAP by nuclear magnetic resonance Spectroscopy and
555 functional studies on GH release and gene expression. *Endocrinology* 148:5042-5059
- 556 31. **Wong AO, Ng S, Lee EK, Leung RC, Ho WK** 1998 Somatostatin inhibits (D-Arg⁶, Pro⁹-NET)
557 salmon gonadotropin-releasing hormone- and dopamine D1-stimulated growth hormone release
558 from perfused pituitary cells of Chinese grass carp, *Ctenopharyngodon idellus*. *Gen Comp*
559 *Endocrinol* 110:29-45
- 560 32. **Jiang Q, Ko WK, Wong AO** 2011 Insulin-like growth factor as a novel stimulator for somatolactin
561 secretion and synthesis in carp pituitary cells via activation of MAPK cascades. *Am J Physiol*
562 *Endocrinol Metab* 301:E1208-1219
- 563 33. **Wong AO, Cheung HY, Lee EK, Chan KM, Cheng CH** 2002 Production of recombinant goldfish
564 prolactin and its applications in radioreceptor binding assay and radioimmunoassay. *Gen Comp*
565 *Endocrinol* 126:75-89
- 566 34. **Jiang Q, Wong AO** 2013 Signal transduction mechanisms for autocrine/paracrine regulation of
567 somatolactin α secretion and synthesis in carp pituitary cells by somatolactin α and β . *Am J Physiol*
568 *Endocrinol Metab* 304:E176-186
- 569 35. **Wong AO, Li W, Leung CY, Huo L, Zhou H** 2005 Pituitary adenylate cyclase-activating
570 polypeptide (PACAP) as a growth hormone (GH)-releasing factor in grass carp. I. Functional
571 coupling of cyclic adenosine 3',5'-monophosphate and Ca²⁺/calmodulin-dependent signaling
572 pathways in PACAP-induced GH secretion and GH gene expression in grass carp pituitary cells.
573 *Endocrinology* 146:5407-5424
- 574 36. **Martinez A, Treston AM** 1996 Where does amidation take place? *Mol Cell Endocrinol* 123:113-
575 117
- 576 37. **Almeida TA, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martin JD, Candenias ML** 2004
577 Tachykinins and tachykinin receptors: Structure and activity relationships. *Curr Med Chem*
578 11:2045-2081
- 579 38. **Shimizu Y, Matsuyama H, Shiina T, Takewaki T, Furness JB** 2008 Tachykinins and their

580 functions in the gastrointestinal tract. *Cell Mol Life Sci* 65:295-311

581 39. **Page NM, Woods RJ, Gardiner SM, Lomthaisong K, Gladwell RT, Butlin DJ, Manyonda IT,**
582 **Lowry PJ** 2000 Excessive placental secretion of neurokinin B during the third trimester causes pre-
583 eclampsia. *Nature* 405:797-800

584 40. **Cintado CG, Pinto FM, Devillier P, Merida A, Candenas ML** 2001 Increase in neurokinin B
585 expression and in tachykinin NK3 receptor-mediated response and expression in the rat uterus with
586 age. *J Pharmacol Exp Ther* 299:934-938

587 41. **Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, Martin JD, Flores C, Mantecon**
588 **AG, Story ME, Pinto FM** 2003 Tachykinins and tachykinin receptors in human uterus. *Brit J*
589 *Pharmacol* 139:523-532

590 42. **Pinto FM, Almeida TA, Hernandez M, Devillier P, Advenier C, Candenas ML** 2004 mRNA
591 expression of tachykinins and tachykinin receptors in different human tissues. *Eur J Pharmacol*
592 494:233-239

593 43. **Ravina CG, Seda M, Pinto FM, Orea A, Fernandez-Sanchez M, Pintado CO, Candenas ML**
594 2007 A role for tachykinins in the regulation of human sperm motility. *Hum Reprod* 22:1617-1625

595 44. **Kawauchi H, Sower SA, Moriyama S** 2009 The neuroendocrine regulation of prolactin and
596 somatolactin secretion in fish. *Fish Physiol* 28:197-234

597 45. **Zhu Y, Stiller JW, Shaner MP, Baldini A, Scemama JL, Capehart AA** 2004 Cloning of
598 somatolactin α and β cDNAs in zebrafish and phylogenetic analysis of two distinct somatolactin
599 subtypes in fish. *J Endocrinol* 182:509-518

600 46. **Zhu Y, Song D, Tran NT, Nguyen N** 2007 The effects of the members of growth hormone family
601 knockdown in zebrafish development. *Gen Comp Endocrinol* 150:395-404

602 47. **Jiang Q, He ML, Wang XY, Wong AOL** 2008 Grass carp somatolactin: II. Pharmacological study
603 on postreceptor signaling mechanisms for PACAP-induced somatolactin α and β gene expression.
604 *Am J Physiol Endocrinol Metab* 295:E477-E490

605 48. **Klausen C, Severson DL, Chang JP, Habibi HR** 2005 Role of PKC in the regulation of
606 gonadotropin subunit mRNA levels: Interaction with two native forms of gonadotropin-releasing
607 hormone. *Am J Physiol Regul Integr Comp Physiol* 289:R1634-R1643

- 608 49. **Newton AC** 2001 Protein kinase C: Structural and spatial regulation by phosphorylation, cofactors,
609 and macromolecular interactions. *Chem Rev* 101:2353-2364
- 610 50. **Garcia M, Sakamoto K, Shigekawa M, Nakanishi S, Ito S** 1994 Multiple mechanisms of
611 arachidonic acid release in Chinese hamster ovary cells transfected with cDNA of substance P
612 receptor. *Biochem Pharmacol* 48:1735-1741
- 613 51. **Taylor CW, Tovey SC, Rossi AM, Lopez Sanjurjo CI, Prole DL, Rahman T** 2014 Structural
614 organization of signalling to and from IP₃ receptors. *Biochem Soc Trans* 42:63-70
- 615 52. **Vela J, Perez-Millan MI, Becu-Villalobos D, Diaz-Torga G** 2007 Different kinases regulate
616 activation of voltage-dependent calcium channels by depolarization in GH₃ cells. *Am J Physiol Cell*
617 *Physiol* 293:C951-959
- 618
619

620 **Legends**

621

622 Fig.1. Sequence analysis, genomic Southern and tissue distribution of grass carp TAC3. (A) Protein
623 sequence alignment of grass carp TAC3 with that of other vertebrates using Clustal-W algorithm with
624 MacVector program. The conserved a.a. residues are boxed in grey and the dibasic protein cleavage
625 sites (KR & GRR) are marked with inverted triangles. (B) Phylogenetic analysis of TAC3 nucleotide
626 sequences using the neighbor-joining method with MEGA 6.0. The numbers presented in the guide-
627 tree are the percentage of bootstrap values based on 1000 bootstraps. Ciona TAC3, a representative of
628 the invertebrate sequence, was used as an out-group. (C) Southern blot of carp TAC3. Genomic DNA
629 was isolated from whole blood of grass carp, digested with restriction enzymes as indicated, resolved
630 by agarose gel electrophoresis, and subjected to Southern blot by hybridization with a DIG-labeled
631 cDNA probe for carp TAC3. (D) Tissue expression profile of carp TAC3. Total RNA was isolated
632 from selected tissues and brain areas in grass carp and subjected to RT-PCR using primers specific for
633 TAC3 (TGTCAGCAGTCAGAGTCTCAAAG & AACCCACGACGAAACCTCAGT). PCR reaction
634 was fixed at 40 cycles with 30 sec at 94°C for denaturing, 30 sec at 56 °C for annealing and 30 sec at
635 72 °C for extension. Authenticity of PCR products was confirmed by Southern blot using the DIG-
636 labeled TAC3 probe and parallel RT-PCR for β -actin was used as the internal control.

637

638 Fig.2. Effects of TAC3 gene products on $SL\alpha$ and PRL synthesis and secretion in carp pituitary cells.
639 Time course of grass carp NKB (1 μ M) and NKBRP treatment (1 μ M) on (A) $SL\alpha$ and PRL secretion,
640 cell content and total production, and (B) $SL\alpha$ and PRL mRNA expression in carp pituitary cells. (C)
641 Dose-dependence of 24-hr treatment with increasing levels of NKB and NKBRP (0.1-1000 nM) on
642 $SL\alpha$ and PRL secretion and mRNA expression. After drug treatment, culture medium was harvested
643 for measurement of hormone release and cell lysate was prepared for monitoring hormone content in
644 pituitary cells. In parallel experiments, total RNA was isolated for real-time PCR of $SL\alpha$ and PRL
645 mRNA using primers specific for the respective gene targets (ACCCACTGTACTTCAATCTCC &
646 CGTCGTAACGATCAAGAGTAG for $SL\alpha$ and CTCAGCACCTCTCTACCAATGACC & GCGG
647 AAGCAGGACAACAGAAAATG for PRL). Real-time PCR was routinely performed for 35 cycles

648 with denaturation at 94 °C for 30 sec, annealing at 52 °C for SL α or 59 °C for PRL for 30 sec, and
649 extension at 72 °C for 30 sec. In the data presented (Mean \pm SEM), the groups denoted by different
650 letters represent a significant difference at P < 0.05 (ANOVA followed by Dunnett's test).

651

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

671

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

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

681

682 Fig.5. Functional role of PLC-dependent pathway in pituitary regulation of SL α and PRL by TAC3
683 gene products. Effects of 24-hr co-treatment with the PLC inhibitor U073122 (10 μ M), PKC inhibitor
684 GF109203X (20 μ M) or IP₃ receptor blocker 2-APB (100 μ M) on NKB (1 μ M)- and NKBRP (1 μ M)-
685 induced (A) SL α and (B) PRL secretion and mRNA expression in carp pituitary cells. After drug
686 treatment, culture medium was harvested for hormone release and total RNA was extracted from the
687 remaining cells for real-time PCR of the respective gene targets.

688

689 Fig.6. Functional coupling of TAC3 gene products with Ca²⁺ signaling in carp pituitary cells. (A)
690 Effects of NKB (1 μ M) and NKBRP (1 μ M) on intracellular Ca²⁺ levels in carp pituitary cells. Parallel
691 treatment with the vehicle (Veh) used for dissolving the TAC3 gene products was used as the solvent
692 control. Effects of (B) co-treatment with the VSCC blocker Nifedipine (10 μ M, Nifed) or (C) removal
693 of extracellular Ca²⁺ using a Ca²⁺-free medium on Ca²⁺ signals triggered by NKB (1 μ M) and NKBRP
694 (1 μ M) in carp pituitary cells. (D) Effects of co-treatment with the IP₃ receptor blocker 2-APB (100 μ M)
695 on NKB (1 μ M) and NKBRP (1 μ M)-induced Ca²⁺ responses in pituitary cells incubated with the
696 Ca²⁺-free medium. (E) Effects of increasing doses of the Ca²⁺ ionophore A23187 (0.1 - 100 nM, 24 hr)
697 on SL α and PRL release and mRNA expression in carp pituitary cells. In the experiments for Ca²⁺
698 measurement, pituitary cells were pre-loaded with the Ca²⁺-sensitive dye Fura-2 and Ca²⁺ data were
699 presented as a ratio of the fluorescence emission obtained with excitation at 340 nm and 380 nm,
700 respectively (as "F340/F380 Ratio"). For the studies on SL α and PRL secretion and gene expression,
701 culture medium was harvested after drug treatment for hormone release and total RNA was extracted
702 from pituitary cells for real-time PCR of the respective gene targets.

703

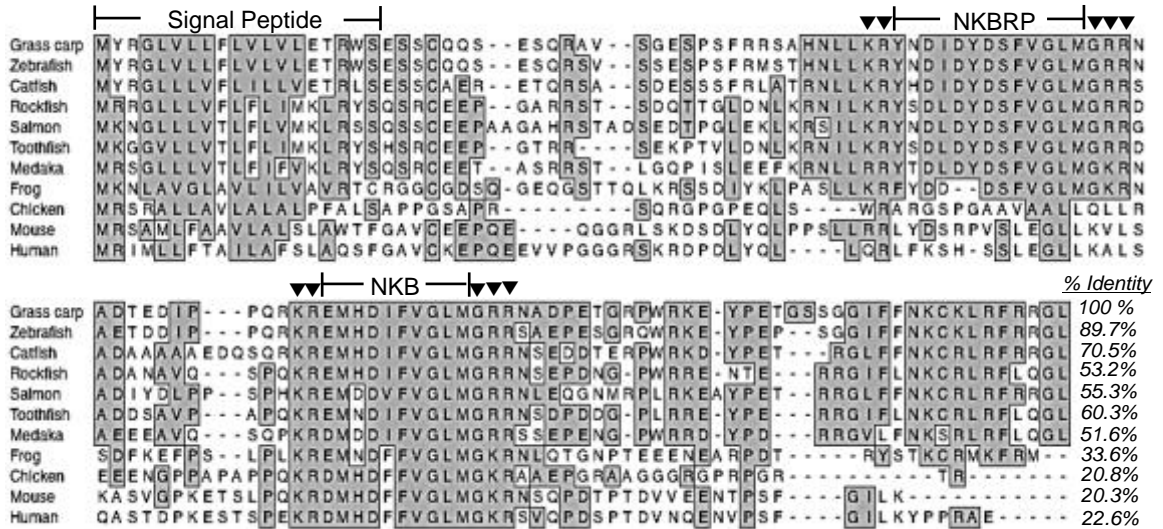
704 Fig.7. Functional role of Ca^{2+} -dependent pathway in pituitary regulation of $\text{SL}\alpha$ and PRL by TAC3
705 gene products. Effects of 24-hr incubation with Ca^{2+} -free medium or co-treatment with the VSCC
706 blocker Nifedipine (10 μM), CaM antagonist calmidazolium (1 μM) or CaMK-II inactivator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

711

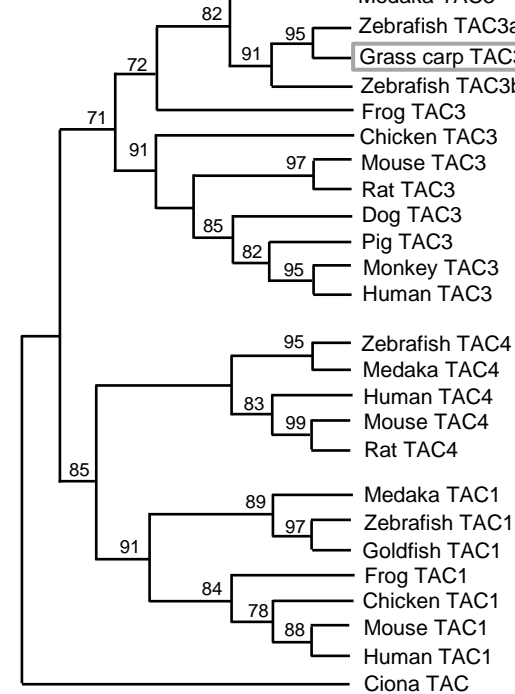
712 Fig.8. Working model of NKB and NKBRP induction of $\text{SL}\alpha$ and PRL synthesis and secretion in
713 carp pituitary cells. In grass carp, two mature peptides, NKB and NKBRP, can be produced from TAC3
714 preprohormone, presumably by protein processing via the two dibasic cleavage sites (KR & GRR)
715 flanking the respective gene products. These two TAC3 gene products through differential activation
716 of NK2R expressed in PRL cells and NK3R expressed in $\text{SL}\alpha$ cells can up-regulate PRL and $\text{SL}\alpha$
717 transcript expression, protein production and hormone secretion in the respective cell types within the
718 carp pituitary. These stimulatory effects, except for a lack of PKC involvement in the PRL responses,
719 appear to be mediated by the AC/cAMP/PKA, PLC/IP₃/PKC and Ca^{2+} /CaM/CMK-II cascades.

Fig.1

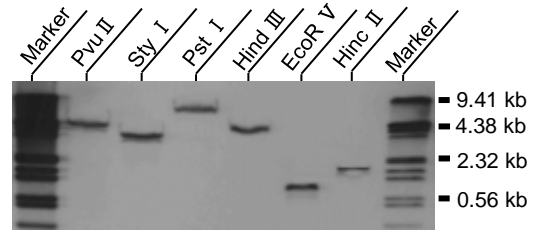
A



B



C



D

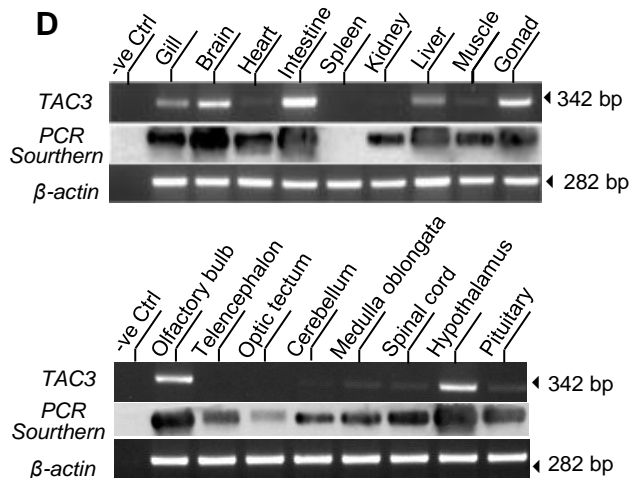


Fig.2

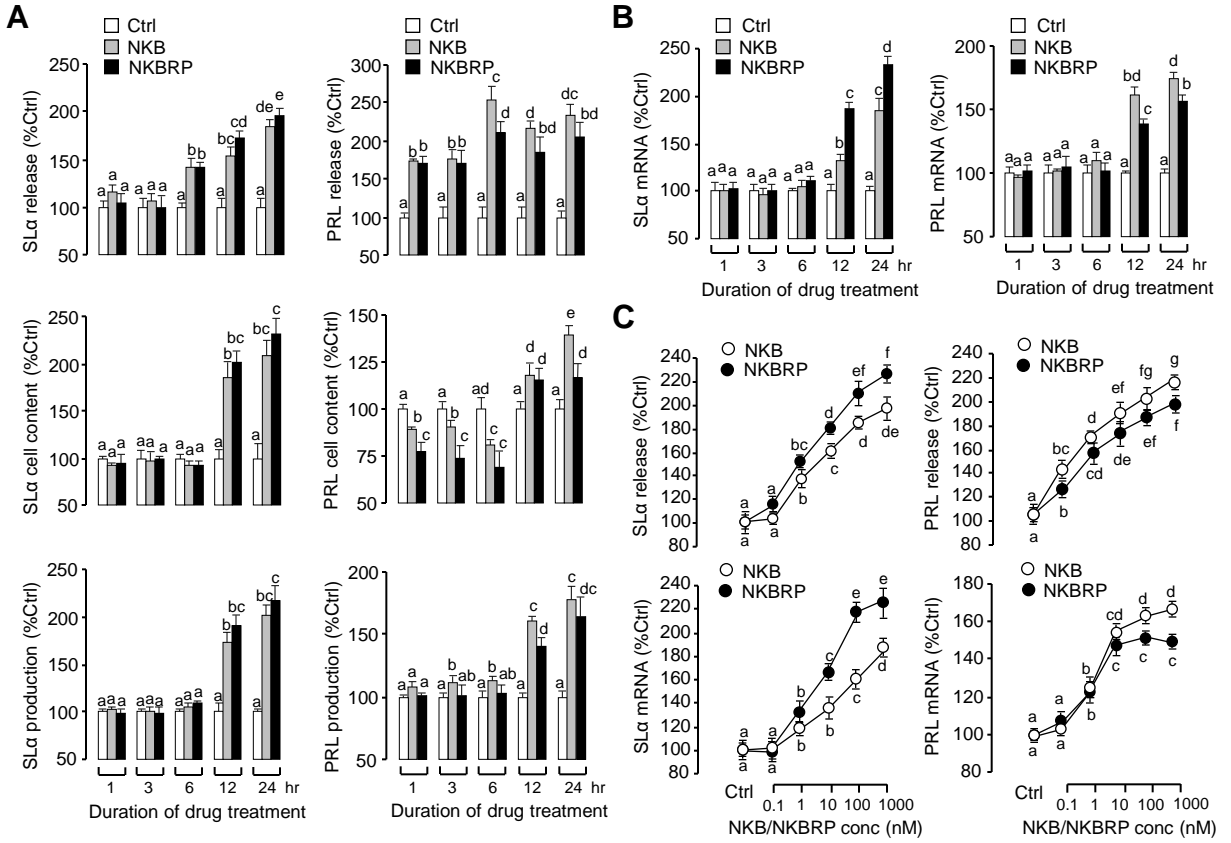


Fig.3

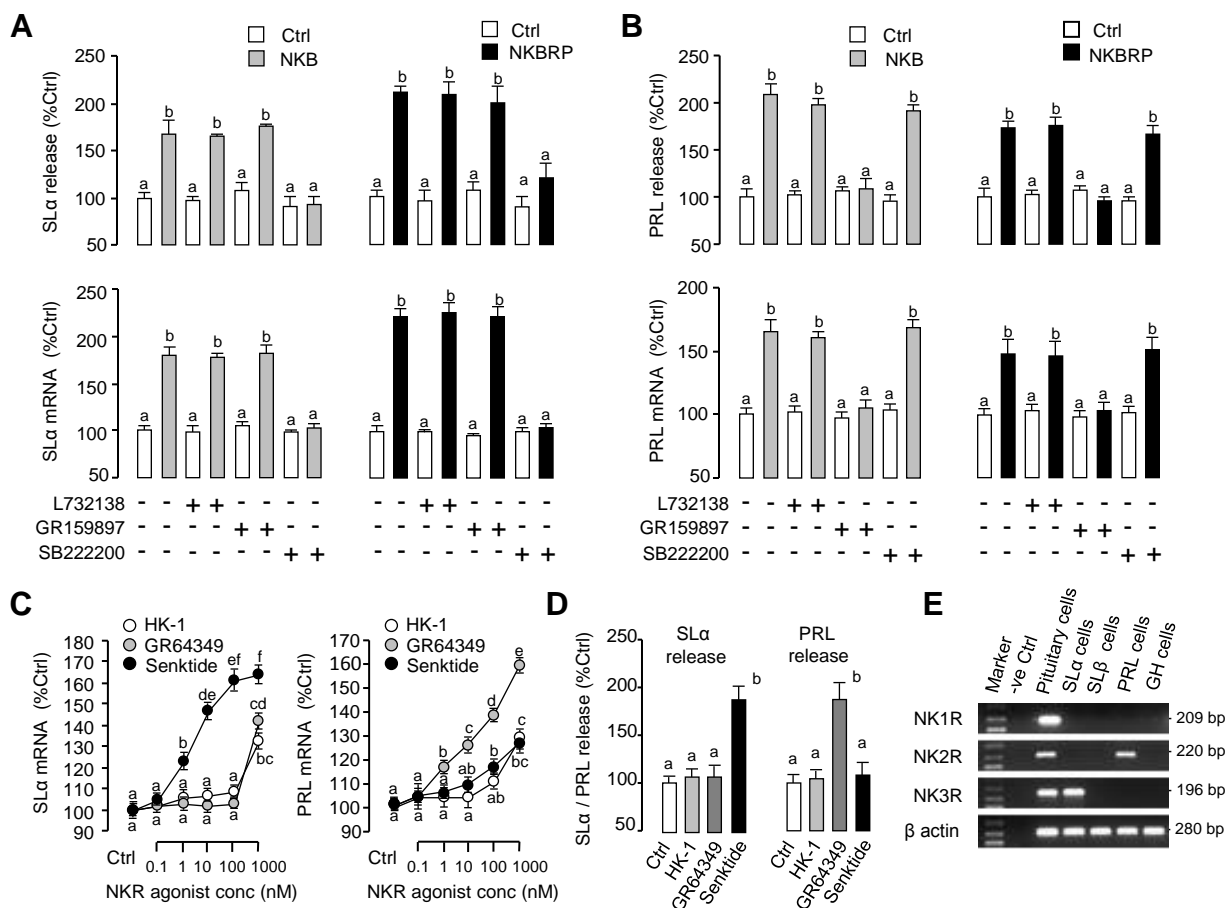


Fig.4

[Click here to download Figure: Fig-4.pptx](#)

Fig.4

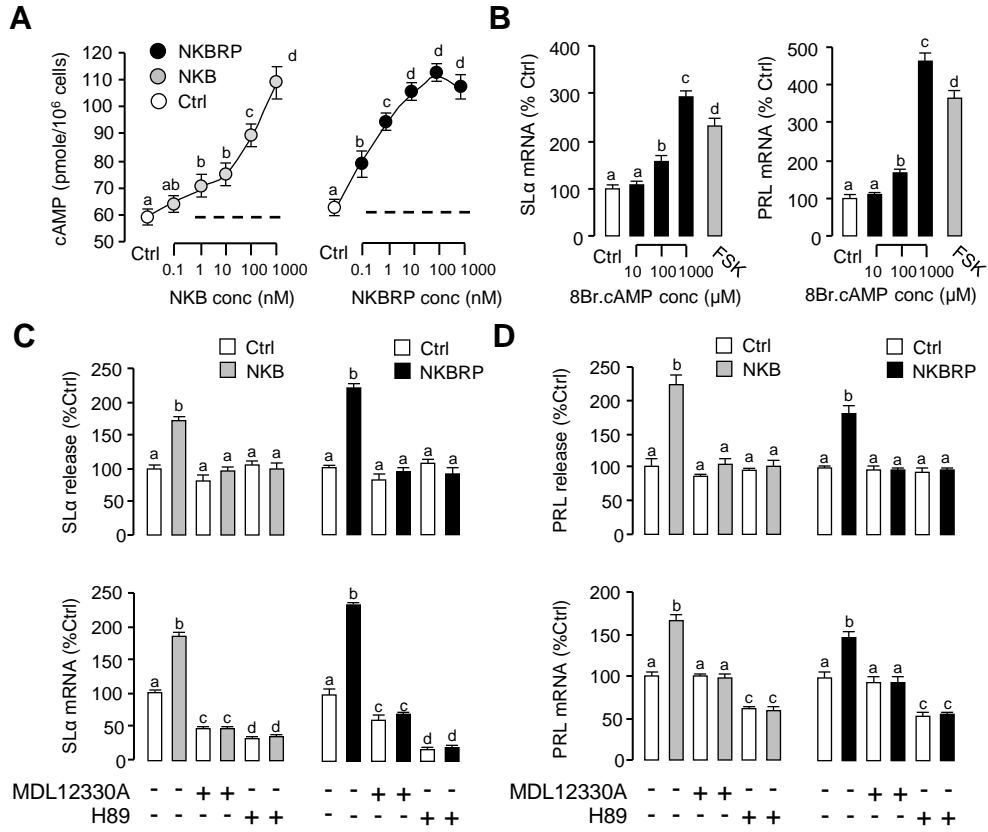


Fig.5

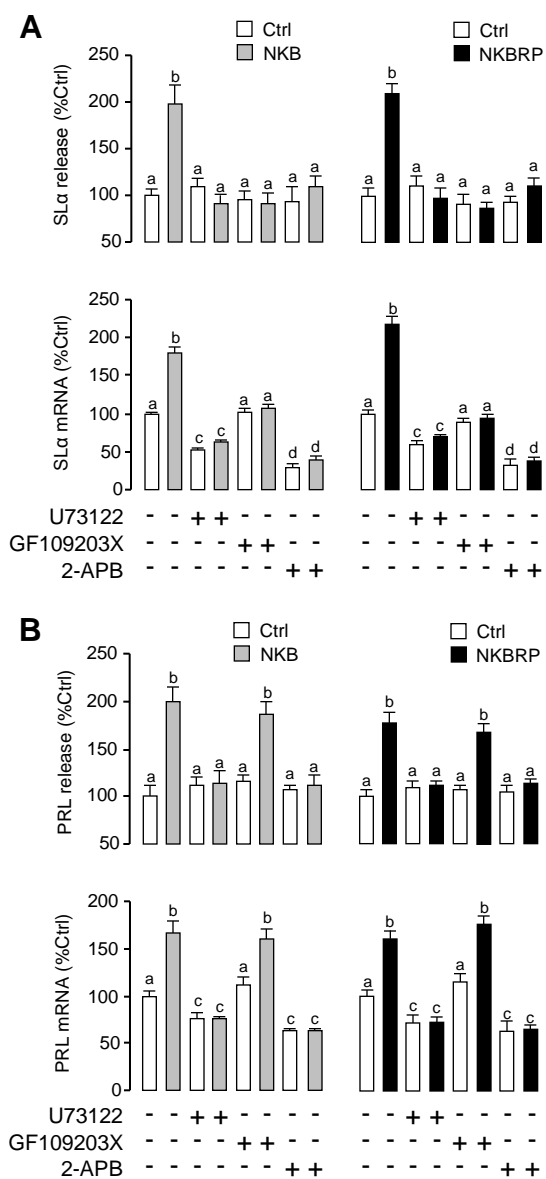


Fig.6

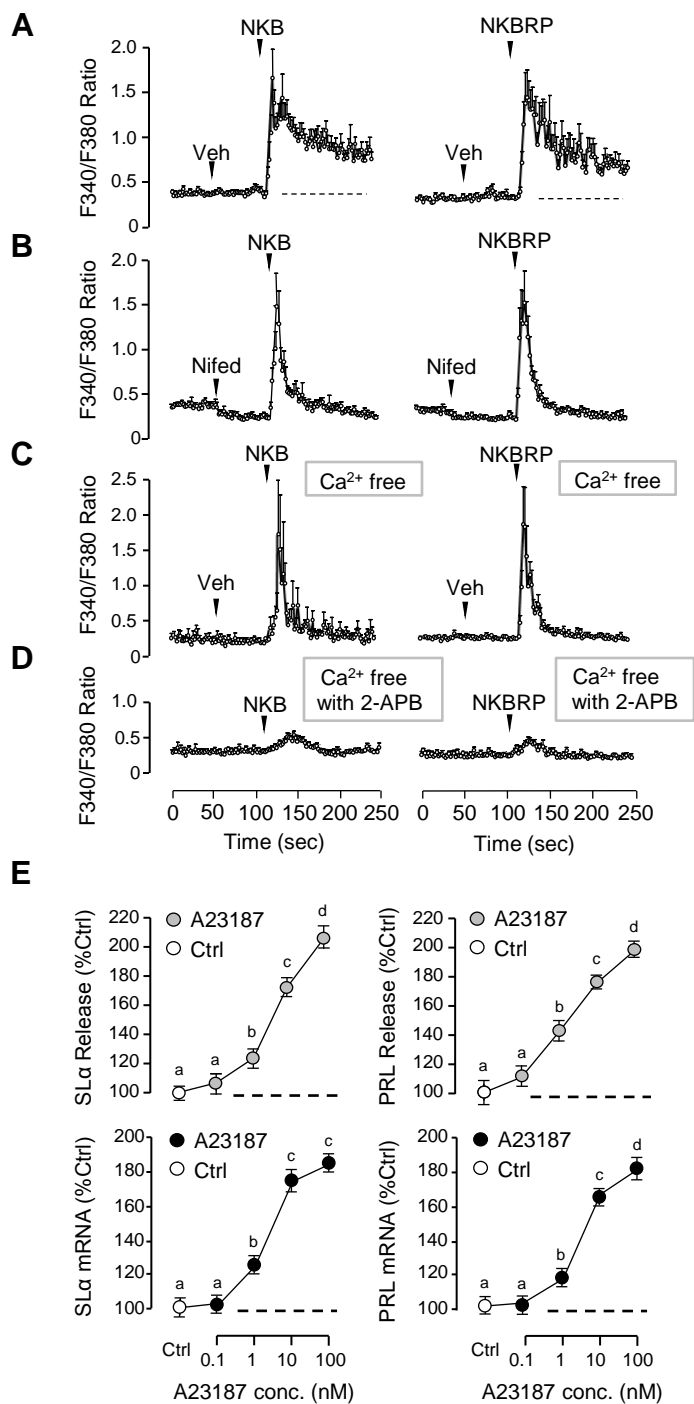


Fig.7

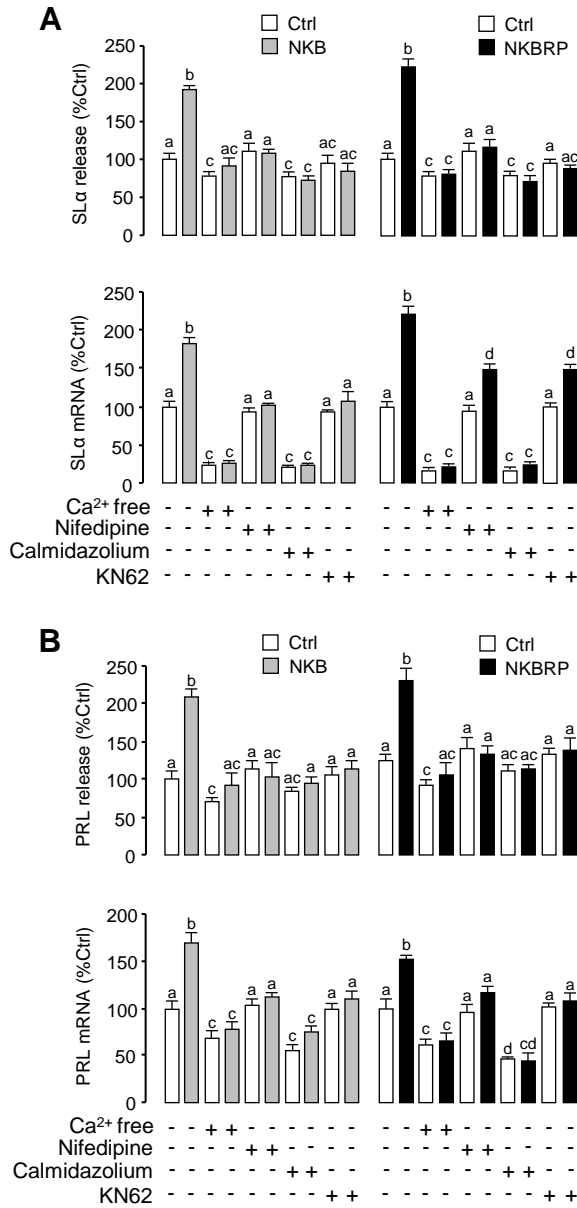
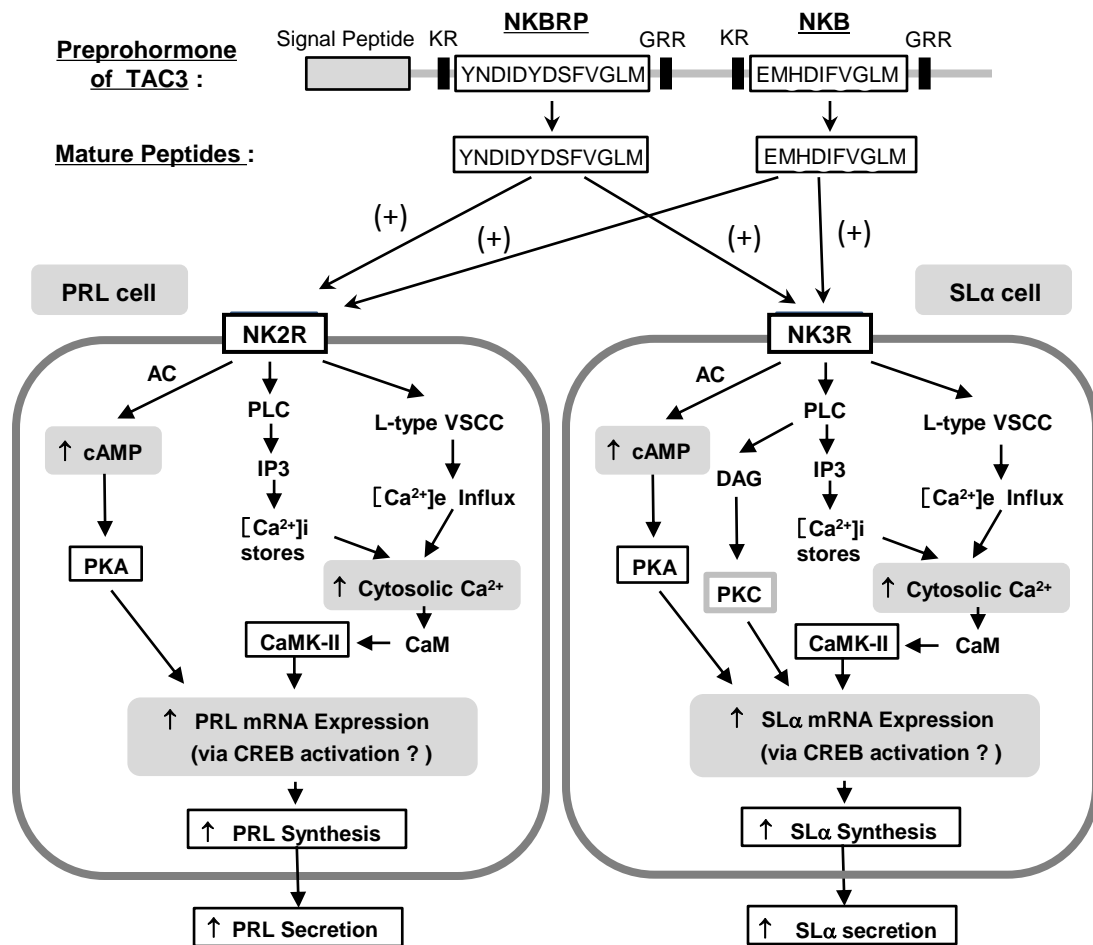


Fig.8

Working model



Supplemental Fig.1

[Click here to download Supplemental Material: Supplemental_Fig-1.pptx](#)

Supplemental Fig.2

[Click here to download Supplemental Material: Supplemental_Fig-2.pptx](#)

Supplemental Fig.3

[Click here to download Supplemental Material: Supplemental_Fig-3.pptx](#)

Supplemental Fig.4

[Click here to download Supplemental Material: Supplemental_Fig-4.pptx](#)

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 RRITVEEALAHPLYEQ 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