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Abstract: In mammals, neurokinin B (NKB), the gene product of the tachykinin family member TAC3, is known to be a key regulator for episodic release of luteinizing hormone (LH). Its regulatory actions are mediated by a subpopulation of kisspeptin neurons within the arcuate nucleus with co-expression of NKB and dynorphin A (commonly called the "KNDy neurons"). By forming an autosynaptic feedback loop within the hypothalamus, the KNDy neurons can modulate gonadotropin-releasing hormone (GnRH) pulsatility and subsequent LH release in the pituitary. NKB regulation of LH secretion has been recently demonstrated in zebrafish, suggesting that the reproductive functions of NKB may be conserved from fish to mammals. Interestingly, the TAC3 genes in fish not only encode the mature peptide of NKB but also a novel tachykinin-like peptide, namely NKB-related peptide (or neurokinin F). Recent studies in zebrafish also reveal the neuroanatomy of TAC3/kisspeptin system within the fish brain is quite different from that of mammals. In this article, the current ideas of "KNDy neuron" model for GnRH regulation and steroid feedback, other reproductive functions of NKB including its local actions in the gonad and placenta, the revised model of tachykinin evolution from invertebrates to vertebrates, as well as the emerging story of the two TAC3 gene products in fish, NKB and NKB-related peptide, will be reviewed with stress on the areas with interesting questions for future investigations.

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Neurokinin B and Reproductive Functions: - “KNDy Neuron”

Model in Mammals and the Emerging Story in Fish.

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Running Title: NKB and Reproduction

Highlights:

- Current ideas on the reproductive functions of neurokinin B in mammals.
- Revised model of tachykinin evolution based on sea anemone sequences.
- Recent findings of neurokinin B and neurokinin B-related peptide in fish.

Key Words: Neurokinin B; Kisspeptin; Gonadotropin-Releasing Hormone; KNDy Neurons; Neurokinin Receptor; Reproduction

Abbreviations: NKB, neurokinin B; Dyn, dynorphin; NKBRP, NKB-related peptide; SP, substance P; NKA, neurokinin A; HK-1, hemokinin-1; endokinin, EK; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; SL, somatolactin; ER, estrogen receptor; NKR, neurokinin receptor; NK1R, NK1 receptor; NK2R, NK2 receptor; NK3R, NK3 receptor; KOR, kappa opioid receptor; PKC, protein kinase C; PKA, protein kinase A; AC, adenylyl cyclase; PLC, phospholipase C; PI, phosphatidylinositol; IP₃, inositol triphosphate; [Ca²⁺]_i, intracellular Ca²⁺; [Ca²⁺]_e, extracellular Ca²⁺; PLD, phospholipase D; CaM, calmodulin; CaMK-II, Ca²⁺/calmodulin-dependent protein kinase-II; MAPK, mitogen-activated protein kinase; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; vmARC, ventromedial arcuate nucleus; POA, preoptic area; NVT, nucleus ventralis tuberis; NRL, nucleus recessus lateralis; RCh, retrochiasmatic area; I.P., intraperitoneal injection; I.C.V., intracerebroventricular injection; OVX, ovariectomy; CL, corpora lutea; NEP, neprilysin; NEP-2, neprilysin-2

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45 **Abstract**

46

47 In mammals, neurokinin B (NKB), the gene product of the tachykinin family member
48 TAC3, is known to be a key regulator for episodic release of luteinizing hormone (LH). Its
49 regulatory actions are mediated by a subpopulation of kisspeptin neurons within the arcuate
50 nucleus with co-expression of NKB and dynorphin A (commonly called the “KNDy neurons”).
51 By forming an “autosynaptic feedback loop” within the hypothalamus, the KNDy neurons can
52 modulate gonadotropin-releasing hormone (GnRH) pulsatility and subsequent LH release in
53 the pituitary. NKB regulation of LH secretion has been recently demonstrated in zebrafish,
54 suggesting that the reproductive functions of NKB may be conserved from fish to mammals.
55 Interestingly, the TAC3 genes in fish not only encode the mature peptide of NKB but also a
56 novel tachykinin-like peptide, namely NKB-related peptide (or neurokinin F). Recent studies
57 in zebrafish also reveal the neuroanatomy of TAC3/kisspeptin system within the fish brain is
58 quite different from that of mammals. In this article, the current ideas of “KNDy neuron”
59 model for GnRH regulation and steroid feedback, other reproductive functions of NKB
60 including its local actions in the gonad and placenta, the revised model of tachykinin
61 evolution from invertebrates to vertebrates, as well as the emerging story of the two TAC3
62 gene products in fish, NKB and NKB-related peptide, will be reviewed with stress on the
63 areas with interesting questions for future investigations.

64

65 **1. Introduction**

66

67 In mammals, tachykinins including substance P (SP), neurokinin A (NKA), neurokinin B
68 (NKB), hemokinin-1 (HK-1) and various forms of endokinins (EKs) represent a major group
69 of brain/gut peptides with important functions as neurotransmitters, endocrine hormones and
70 local autocrine/paracrine regulators (Satake et al., 2013). Multiple tachykinin genes with
71 different gene products, including TAC1 encoding SP and NKA, TAC3 (also referred to as
72 TAC2 in rodents) encoding NKB and TAC4 encoding HK-1 and EKs, have been reported
73 (Pennefather et al., 2004a) and probably are the result of gene duplication occurred during
74 vertebrate evolution (Conlon and Larhammar, 2005). The peptide products of tachykinin
75 genes (except for EKC and EKD) all share a common C-terminal α -amidated motif “FXGLM”
76 (also called the “message domain”, with X represents a hydrophobic residue with aromatic or
77 branched aliphatic side chain) which is critical for receptor binding and bioactivities (Almeida
78 et al., 2004). Tachykinins are widely expressed at the tissue level, with the gene products of
79 TAC1 and TAC3 detected mainly in neuronal structures within the CNS (Satake and Kawada,
80 2006) and TAC4 products in non-neuronal tissues in the periphery (e.g., in spleen, stomach,
81 lung, bone marrow, thymus, lymph nodes, prostate and uterus) (Page, 2004). The gene
82 products of tachykinin family are known to be involved in nonadrenergic and noncholinergic
83 (NANC) neurotransmission within the CNS (Almeida et al., 2004), nociceptive functions

84 mediated via the spinal cord (Patte-Mensah et al., 2005), smooth muscle activity related to
85 airway opening (Mizuta et al., 2008), vasodilation/tuning of blood pressure (Abdelrahman and
86 Pang, 2005) and gut motility (Lecci et al., 2006), fluid secretion in intestinal epithelium
87 (Shimizu et al., 2008), immunomodulation (Zhang et al., 2006) and neuroendocrine regulation
88 of reproductive functions (Wang and Tian, 2012). Their malfunctions can be linked with
89 clinical conditions including inflammatory bowel syndrome, rheumatoid arthritis, bronchial
90 asthma, hypertension, chronic pain, Alzheimer's disease, depression and schizophrenia (Lecci
91 and Maggi, 2003).

92

93 The physiological functions of tachykinins are mediated via three subtypes of neurokinin
94 receptors (NKR), namely NK1 receptor (NK1R), NK2 receptor (NK2R) and NK3 receptor
95 (NK3R), which are members of the rhodopsin-type class I group G-protein coupled receptors
96 (Satake et al., 2013). Apparently, the random structure of tachykinins **can adopt** a helical
97 configuration (mainly in the central core & C-terminal) when the peptide is present in close
98 proximity to the plasma membrane of target cells (Grace et al., 2003). Subsequent binding
99 via the C-terminal "FXGLM" motif of tachykinins with TMD6 and TMD7 of the binding
100 pocket of the respective receptors, e.g., NKB binding with NK3R (Ganjiwale et al., 2011),
101 presumably constitutes a major step to trigger post- receptor signaling via G_o and/or $G_{q/11}$
102 (Khawaja and Rogers, 1996; Quartara and Maggi, 1997) followed by cAMP production

103 (Lecat et al., 2002; Palanche et al., 2001), PLC-dependent PI hydrolysis (Mizuta et al., 2008;
104 Nakajima et al., 1992), mobilization of IP₃-sensitive [Ca²⁺]_i stores (Mizuta et al., 2008),
105 [Ca²⁺]_e entry via voltage-dependent Ca²⁺ channels (Khawaja and Rogers, 1996; Mau et al.,
106 1997), nNOS-mediated NO and cGMP production (Linden et al., 2000), MAPK activation
107 associated with NKR internalization (Alblas et al., 1996; DeFea et al., 2000), and PLD
108 activation (Torrens et al., 1998) coupled with downstream arachidonic acid release (Garcia et
109 al., 1994). Individual subtypes of NKR, probably via differential interactions with the
110 C-terminal of tachykinins (Satake et al., 2003), are known to exhibit differential selectivity for
111 various members of tachykinins, with NK1R preferring SP (SP > NKA > NKB), NK2R
112 preferring NKA (NKA > NKB > SP) and NK3R preferring NKB (NKB > NKA > SP),
113 respectively (Almeida et al., 2004). With potentials for therapeutic use in human diseases,
114 the structure-activity relationship for ligand/receptor interaction and rational design of
115 agonist/antagonist for different NKR subtypes have been a major focus for tachykinin
116 research (Ganjiwale and Cowsik, 2013).

117

118 In recent years, the gene product of TAC3, namely NKB, has emerged as a key regulator
119 for reproductive functions in mammals, especially in the control of GnRH pulsatility within
120 the hypothalamus (Goodman et al., 2013a; Goodman et al., 2013b). The functional role of
121 TAC3 in reproduction has also been implicated in fish model, mainly based on the recent

122 reports in zebrafish (Biran et al., 2012; Ogawa et al., 2012). In this article, the current model
123 for NKB modulation of GnRH pulsatility, other aspects of NKB in reproductive functions as
124 well as the current ideas of NKB evolution and the emerging story of the TAC3 gene products
125 in fish reproduction and pituitary functions will be reviewed with stress on various areas with
126 questions remained to be answered.

127

128 **2. KNDy neurons and GnRH pulse generator**

129

130 The interest on TAC3 involvement in reproductive function was first initiated by the recent
131 demonstration that loss-of-function mutations in NKB (e.g., M10T mutation) or NK3R (e.g.,
132 G93D, I249V, Y256H, Y315C & P353S mutations) can lead to familial hypogonadotropic
133 hypogonadism or even infertility in human (Guran et al., 2009; Topaloglu et al., 2009; Young
134 et al., 2010). Together with the findings that prepubertal increases in hypothalamic NKB and
135 NK3R expression can be used as the markers for pubertal activation during sexual maturation,
136 e.g., in rat (Navarro et al., 2012b) and mouse (Gill et al., 2012), and disruption in NKB/NK3R
137 system tends to postpone/inhibit puberty in human (Topaloglu et al., 2010) and animal models,
138 (e.g., delaying vaginal opening in female rat) (Navarro et al., 2012a; Topaloglu, 2010), it is
139 commonly accepted that NKB signaling serves as a gatekeeper for puberty onset as well as a
140 key modulator for normal functioning of reproductive system in adulthood (Topaloglu, 2010).

141 Although the role of NKB in puberty onset is still an area of active research and inconsistency
142 has been reported, e.g., in sheep model with notable increases in NKB immunoreactivity and
143 kisspeptin neuronal population in the hypothalamus only after but not before puberty (Nestor
144 et al., 2012), a common consensus has been reached regarding the reproductive functions of
145 NKB in adult by acting through the hypothalamo-pituitary-gonadal axis and the mechanisms
146 mainly involve the neuroendocrine regulation of GnRH pulsatility within the hypothalamus
147 via a subpopulation of Kisspeptin neurons located within the arcuate nuclei (ARC) with co-
148 expression of NKB and Dynorphin A (commonly referred to as the KNDY neurons) (Lehman
149 et al., 2010; Navarro, 2012).

150

151 In the past decades, kisspeptin, through activation of its cognate receptor GPR54 expressed
152 in GnRH neurons, has emerged as an upstream stimulator for GnRH secretion and constitutes
153 a new regulatory target for steroid feedback in the hypothalamo-pituitary-gonadal axis
154 (Dungan et al., 2006). Based on the studies in rodents, sheep, and to a lesser extent in monkey,
155 the current model for KNDy regulation of GnRH release (Fig.1) involves (i) a network of
156 KNDy neurons that **are profusely interconnected in the ARC** and commonly believed to form
157 bilateral interconnections/autosynaptic contact within the neuronal population (Krajewski et
158 al., 2010), and (ii) KNDy innervation of GnRH neurons/nerve fibers in the preoptic nuclei
159 (POA) **and median eminence** of the hypothalamus (Navarro, 2013). Apparently, local release

160 of NKB can activate NK3R expressed in KNDy neurons (Amstalden et al., 2010; Billings et
161 al., 2010) and subsequent increase in kisspeptin signal to POA stimulates GnRH neuronal
162 activity (Wakabayashi et al., 2013) and triggers GnRH secretion in the median eminence of
163 the hypothalamus (Ramaswamy et al., 2010), which can then exert its reproductive functions
164 by regulating pulsatile release of LH from the pituitary (Goodman et al., 2013a). Besides
165 NK3R, which is abundantly expressed in KNDy neurons (Amstalden et al., 2010), a recent
166 study [in mice](#) using antagonists for the respective receptor subtypes has demonstrated that
167 NK1R and NK2R are also involved in NKB activation of kisspeptin neurons within the ARC
168 (de Croft et al., 2013). However, NK1R and NK2R expression in KNDy neurons in ARC [or](#)
169 [GnRH neurons in POA](#) has not been reported and the post-receptor signaling mechanisms for
170 NKB-induced kisspeptin / GnRH secretion are still unknown.

171

172 Other than the stimulatory signals via NKB, KNDy neurons also exhibit local expression
173 of dynorphin (Dyn) (Burke et al., 2006), which can down-regulate both basal and NKB-
174 [induced](#) neuronal activity in KNDy neurons (e.g., in mice, Ruka et al., 2013) and suppress
175 GnRH (e.g., in OVX ewes, Goodman et al., 1995) and LH release (e.g., in sheep, Goodman et
176 al., 2004; rat, Mostari et al., 2013) presumably by reducing kisspeptin output to GnRH
177 neurons (Goodman et al., 2013b). These inhibitory actions are mediated through activation
178 of κ -opioid receptor (KOR) (de Croft et al., 2013 Mostari et al., 2013) and can be modified by

179 the steroid background of animal model (Ruka et al., 2013). Although NK3R is widely
180 expressed in “Dyn-positive” neurons within the ARC (Burke et al., 2006), KOR expression in
181 KNDy neurons is only marginal/barely detectable (e.g., mouse) (Navarro et al., 2009; Navarro
182 et al., 2011b) and cannot be found in GnRH neurons (e.g., rat) (Mitchell et al., 1997; Sannella
183 and Petersen, 1997), implying that the effects of Dyn on KNDy neurons may be indirect and
184 the involvement of KOR-expressing interneurons is suspected (Fig.1). Although the effect of
185 local release of Dyn on NKB expression/secretion in KNDy neurons is unclear, it is commonly
186 accepted that the functional interplay between the NKB/NK3R and Dyn/KOR systems forms
187 an “autosynaptic feedback” in KNDy neurons within the ARC and the resulting “oscillating
188 output” of kisspeptin may contribute to the pacemaker activity for GnRH pulse generator
189 located within the hypothalamus (Goodman et al., 2013b; Navarro, 2012; Rance et al., 2010).

190

191 Of note, the functional contact of KNDy neurons with GnRH nerve terminals (mainly via
192 varicosities but not synaptic contact) can also be located in the median eminence, especially
193 in the external zone including the lateral palisade area for signal input into hypothalamo-
194 hypophysial portal blood vasculature (Ciofi et al., 2006; Krajewski et al., 2005).
195 Interestingly, GnRH release in this area is sensitive to kisspeptin but not NKB stimulation
196 (Corander et al., 2010), suggesting that (i) the “functional NK3R” may be expressed mainly in
197 the cell bodies of KNDy neurons within ARC but not in their nerve terminals in median

198 eminence, and (ii) the KNDy fibers/nerve terminals in median eminence may be functional
199 only for kisspeptin output from KNDy neurons to trigger GnRH exocytosis into hypophysial
200 portal blood, which may also play a role in synchronization of pulsatile GnRH signals into the
201 pituitary (Choe et al., 2013; Wakabayashi et al., 2013). In rat during lactation, reduced levels
202 of kisspeptin and NKB expression can be observed in the ARC with significant loss of KNDy
203 fibers projecting to GnRH neurons within the POA. In the same model, interestingly, KNDy
204 innervation of GnRH nerve fibers is not affected in the median eminence (True et al., 2011).
205 These findings raise the possibility that the neuronal contact of KNDy and GnRH neurons in
206 the median eminence may represent the major mechanisms for GnRH regulation by kisspeptin
207 during the “negative energy balance” state caused by lactation.

208

209 At present, the “KNDy neuron” model of GnRH regulation has not been fully characterized
210 and inconsistencies of NKB-induced GnRH activation and LH secretion have been reported.
211 For examples, I.P. and I.C.V. injection of NKB did not alter plasma LH levels in male mice
212 (Corander et al., 2010) or OVX rat with estrogen replacement (Grachev et al., 2013) but
213 similar treatment in other studies could consistently induce LH release in male mice through
214 kisspeptin-dependent mechanisms (Garcia-Galiano et al., 2012) and increase LH pulsatility in
215 sheep (Billings et al., 2010) and monkey (Ramaswamy et al., 2010) via NKB/NK3R system.
216 Of note, the LH responses to NKB/NK3R agonist can also be modified by steroid background

217 of the animal (e.g., in rodents). In cyclic female rat or OVX rats with estrogen replacement
218 matched with that of the proestrous phase, brain injection of NK3R agonist could consistently
219 elevate LH levels in circulation whereas a mild inhibition on LH release was noted by similar
220 treatment in OVX rat with estrogen levels reduced to that of the diestrus phase (Navarro et al.,
221 2011). In OVX rat (with/without estrogen replacement), I.C.V. administration of NKB/NK3R
222 agonist was found to inhibit GnRH neuronal activity, down-regulate GnRH gene expression
223 and suppress LH pulses in systemic circulation (Grachev et al., 2012; Kinsey-Jones et al.,
224 2012). Recent studies using the same animal model also showed that the inhibitory effects
225 of NKB on GnRH neurons and LH pulsatility were mediated by KOR activation (Grachev et
226 al., 2012), which is at variance with the blockade of NKB-induced GnRH/LH release by Dyn
227 via KOR reported in the sheep and goat models (Goodman et al., 2013a). Furthermore, NKB
228 treatment in vitro had no effects on GnRH secretion in explant culture of rat hypothalamus
229 (Corander et al., 2010), but similar studies with brain slices of the mouse, intriguingly, reveal
230 that NK3R activation could induce GnRH release (Gaskins et al., 2013) with a parallel rise of
231 neuronal activity in KNDy but not GnRH neurons (Navarro et al., 2011b).

232

233 Although GnRH neurons are known to have little (Krajewski et al., 2005) or no NK3R
234 expression (Amstalden et al., 2010) and in general not considered as a target for NKB
235 (Navarro et al., 2011b), a recent study in GT1-7 GnRH neuronal cells has revealed the

236 presence of NK3R mRNA in this cell line together with GnRH secretion with short-term NKB
237 exposure. Interestingly, prolonged treatment with NKB could inhibit GnRH release in GT1-7
238 cells with a concurrent drop in GnRH gene transcription (Glidewell-Kenney et al., 2013).
239 Whether the inhibitory actions caused by prolonged NK3R activation are also involved in the
240 diversity of GnRH responses reported is not clear, but these biphasic effects of NKB for sure
241 will add to the complexity of “KNDy neuron” model related to [species variation](#), gender
242 difference/steroid background as well as different regulatory targets for NKB within the CNS.
243 It is also worth mentioning that the current studies on the reproductive functions of NKB/
244 NK3R system are focused mainly on GnRH regulation within the hypothalamus. Given that
245 (i) different NKR subtypes are known to be expressed in the pituitary, e.g., NK1R (Larsen et
246 al., 1992) and NK2R in the rat pituitary (Pisera et al., 2003), and (ii) NKB induction of PRL
247 release and enhancement of TRH-induced PRL gene transcription have been reported in rat
248 pituitary cells (Henriksen et al., 1995) and GH₃ lactotroph cell line (Mijiddorj et al., 2012),
249 respectively, the possibility of LH regulation by NKB via direct action at the pituitary level
250 cannot be excluded.

251

252 **3. KNDy neurons and steroid negative feedback**

253

254 Feedback regulation by gonadal steroids represents a key component of the hypothalamo-

255 pituitary-gonadal axis. In the past decades, a major breakthrough in the field of reproductive
256 biology was the identification of kisspeptin as the “missing link” between ovarian output of
257 estrogen and its negative (during follicular phase) and positive feedback (during preovulatory
258 phase) on gonadotropin release during the reproductive cycle (Smith, 2013) (Fig.1). In
259 rodents (e.g., female rat), the differential effects of estrogen on LH secretion are mediated by
260 two clusters of kisspeptin neurons located separately in the ARC and AVPV nuclei of the
261 hypothalamus (Dungan et al., 2006). Both of them have ER α expression and exhibit distinct
262 patterns of kisspeptin expression during the estrous cycle, with kisspeptin mRNA levels
263 reaching its peak during diestrus 2 in ARC followed by a delayed rise to high level in AVPV
264 during proestrus (Adachi et al., 2007). Interestingly, estrogen or testosterone treatment can
265 trigger opposite effects on kisspeptin gene expression in these two nuclei, with stimulation in
266 AVPV but inhibition in ARC (Smith et al., 2007; Smith et al., 2005), which will differentially
267 adjust GnRH neuronal activity via GPR54 activation and lead to the respective positive (by
268 increasing kisspeptin signal from AVPV) and negative effects (by decreasing kisspeptin signal
269 from ARC) on GnRH pulsatility within the hypothalamus (Li et al., 2009). These findings,
270 together with other studies, lead to the conclusions that (i) kisspeptin neurons located in the
271 ARC mediate the negative feedback of estrogen on GnRH neurons and contribute to the LH
272 and FSH inhibitory tone caused by low to medium levels of estrogen commonly observed
273 during the follicular phase of ovarian cycle, and (ii) kisspeptin neurons located in the AVPV,

274 in contrast, are responsible for the positive feedback on GnRH pulse generator by high levels
275 of estrogen during the preovulatory phase, which presumably serves as a major trigger for LH
276 surge and subsequent ovulation (Smith, 2013).

277

278 The system in other mammals (e.g., in sheep without the AVPV area) is also similar and
279 yet distinct from that of the rodents. Apparently, KNDy neurons in the ARC and its role in
280 estrogen negative feedback during the follicular phase of the ovarian cycle are well conserved
281 whereas species variations have been reported for the kisspeptin neurons mediating estrogen
282 positive feedback (Smith, 2013). For examples, kisspeptin neurons in sheep are also located
283 in the dorsolateral POA close to the region with GnRH neurons, and similar to KNDy neurons
284 in ARC, this neuronal population has been confirmed to have ER α expression (Franceschini et
285 al., 2006). In the same animal model, activation of kisspeptin neurons (as reflected by a rise
286 in c-fos expression) in both ARC and POA can be noted during preovulatory phase (Merkley
287 et al., 2012) or in OVX ewes treated with a high dose of estrogen commonly used to trigger
288 positive feedback during LH surge (Smith et al., 2009). These findings raise the possibility
289 that kisspeptin neurons located in these two brain nuclei are both involved in estrogen positive
290 feedback during preovulatory period in sheep. This idea is also consistent with the findings
291 of a recent study using microimplantation of NK3R agonist in the respective nuclei in ewes to
292 cause a notable rise in serum LH levels, suggesting that NKB activation of kisspeptin neurons

293 in ARC and POA may contribute to LH surge during preovulatory phase (Porter et al., 2014).
294 Of note, estrogen treatment is also known to modulate LH secretion via actions in other brain
295 areas, e.g., retrochiasmatic area (RCh) of the hypothalamus (Gallegos-Sanchez et al., 1997).
296 In ewes during anestrous/follicular phase, NK3R expression can be detected in RCh (Billings
297 et al., 2010), and similar to the microimplantation studies in ARC and POA, local activation
298 of NK3R within the RCh area has been shown to induce LH secretion to levels comparable to
299 that observed during LH surge and this stimulatory effect is highly sensitive to the blockade
300 by NK3R antagonist (Porter et al., 2014). Apparently, NKB/NK3R activation of “non-KNDy
301 neurons” outside the ARC and POA areas may also play a role in preovulatory LH surge in
302 the sheep model.

303

304 Unlike GnRH neurons with no steroid receptor expression except for low levels of ER β
305 (Ciofi et al., 1994), the KNDy neurons among the kisspeptin neuronal populations within the
306 ARC are known to express ER α (Franceschini et al., 2006), androgen receptor (Ciofi et al.,
307 1994) and progesterone receptor (Foradori et al., 2002), and play a key role in mediating the
308 negative feedback of estrogen (Mittelman-Smith et al., 2012) as well as progesterone in the
309 gonadotropic axis (Goodman et al., 2004). In mammals (e.g., rat & monkey), ovariectomy
310 in general can up-regulate NKB (Rance and Bruce, 1994) and kisspeptin gene expression in
311 ARC (Rometo et al., 2007). In contrast, estrogen treatment, presumably via ER α activation

312 (Dellovade and Merchenthaler, 2004), can suppress NKB, NK3R and kisspeptin transcript
313 levels in the same area (Gill et al., 2012; Navarro et al., 2011a), which parallel with estrogen
314 inhibition on GnRH neuronal activity and LH release into systemic circulation (Wakabayashi
315 et al., 2010). Of note, I.C.V. injection of Dyn is known to suppress GnRH neuronal activity
316 and LH pulse frequency (e.g., in goat, Wakabayashi et al., 2010) and estrogen inhibition on
317 LH secretion and LH pulsatility can be nullified by co-treatment with KOR antagonists (e.g.,
318 in OVX rat, Mostari et al., 2013). In sheep, attenuation in LH pulses can also be noted with
319 progesterone treatment, and similar to the case of estrogen, the effect is highly sensitive to the
320 blockade by central administration of KOR antagonists (Goodman et al., 2004). Together,
321 these findings provide evidence that the Dyn/KOR system may be involved in the negative
322 feedback by estrogen and progesterone during the reproductive cycle.

323

324 Although it would be logical to assume that the KNDy neurons may serve as the target site
325 within the hypothalamus for negative feedback by gonadal steroids (Navarro, 2012), other
326 studies reported also suggest the otherwise. For examples, endogenous opioids (e.g., in sheep,
327 Goodman et al., 1995) and kisspeptin neurons in ARC (e.g., in mouse, De Croft et al., 2012)
328 were shown to be not involved in estrogen negative feedback and the cause of the discrepancy
329 is unclear but may be related to the use of different approaches or methodologies in individual
330 studies. In mammals, sexual dimorphism of KNDy neurons in the ARC with a notably larger

331 neuronal population size in the female than that of the male is a common phenomenon in the
332 rat (Ruiz-Pino et al., 2012), sheep (Goubillon et al., 2000) and human (Hrabovszky et al., 2011).
333 Besides, differential wiring of KNDy neurons with fibers projecting to the ventromedial ARC
334 (vmARC) and median eminence in the male but with only vmARC innervation in the female
335 has also been mapped in the rat (Ciofi et al., 2006). To date, except for 1-2 reports suggesting
336 a possible role of testosterone in the sheep model (Goubillon et al., 2000; Cheng et al., 2010),
337 the organization effects of gonadal steroids on the sexual dimorphism of KNDy neurons in the
338 hypothalamus have not been elucidated and further investigations are clearly warranted.

339

340 **4. Other aspects of NKB in reproductive functions**

341

342 4.1 *Expression and functions of NKB in the gonad*

343

344 In mammals (e.g., rat), NKB is widely expressed at both transcript level as well as protein
345 level in various components of the reproductive system, e.g., in the placenta (Page et al.,
346 2000), uterus (Cintado et al., 2001; Patak et al., 2003), ovary (Lasaga and Debeljuk, 2011),
347 prostate gland and testis (Pinto et al., 2004). In a recent study on human female genital tract,
348 a detailed anatomical examination has revealed a very close spatial relationship, and in some
349 cases, co-expression of NKB with NK3R and kisspeptin in various areas of the ovary, oviduct

350 and uterus (Cejudo Roman et al., 2012). In testis, NKB and various NKR subtypes can be
351 detected in mature spermatozoa (Pintado et al., 2003), and treatment with NKB, SP and NKA
352 are all effective in stimulating sperm motility, especially the forward progressive movement,
353 via activation of NK1R and NK2R and to a lesser extent through NK3R (Ravina et al., 2007).
354 In human sperm isolated from fresh ejaculate, a zonal distribution of NKB, SP and NKA
355 immunoreactivities has been demonstrated in mature spermatozoa, with SP detected mainly in
356 the acrosome and connecting piece, NKA located in the neck region and to a lower extent in
357 the head and tail region, and NKB distinctly mapped to the equatorial segment of the sperm
358 head (Pinto et al., 2010). Since the equatorial region of sperm head is known to be a key
359 structure for sperm-egg fusion following zonal penetration, the possible role of NKB in the
360 final phase of fertilization, namely the syngamy of male and female gametes, cannot be
361 excluded. In the same study, transcripts of tachykinin-degrading enzymes, namely neprilysin
362 (NEP) and neprilysin-2 (NEP-2), were also detected with NKB, SP and NKA signals in
363 human sperm and inhibiting NEP/NEP-2 activity could also enhance the sperm motility
364 caused by NKR activation, implying that tachykinins are involved in autocrine induction of
365 sperm movement, and probably, their clearance by NEP/NEP-2 may play a role in regulating
366 local activity of tachykinins in mature spermatozoa (Pinto et al., 2010; Ravina et al., 2007).
367 In mammals, SP expression can be located in Leydig cells within the testis (e.g., in rat)
368 (Lasaga and Debeljuk, 2011) and SP treatment in vitro inhibits both basal (Angelova et al.,

369 1991) and LH-induced testosterone production in Leydig cell culture (e.g., in hamster)
370 (Angelova et al., 1996). However, it is still unclear if NKB is also expressed in Leydig cells,
371 and to our knowledge, the functional role of NKB in steroid production has not been
372 examined at the gonad level.

373

374 Unlike the case in testes, NKB expression has been clearly demonstrated in different
375 structural/ functional compartments of the ovary other than the oocyte (Lasaga and Debeljuk,
376 2011; Pintado et al., 2003). In human ovary, co-expression of NKB and NK3R can be
377 detected in both the theca and granulosa cell layers of growing follicles and high levels of
378 their immunostaining signals can also be located in atretic follicles as well as in corpora lutea
379 (CL) (Cejudo Roman et al., 2012). Although the effects of NKB on steroid production in the
380 ovary still remain to be investigated, local production of NKB is believed to play a role in
381 follicular growth/maturation [presumably via NK3R activation](#), as (i) ovarian levels of NKB
382 transcript are significantly reduced in female rat [with sexual acyclicity associated with follicle](#)
383 [senescence induced by hypothyroidism](#) (Ghosh et al., 2007), and (ii) a notable rise in the
384 number of CL and CL cysts can be found in the ovary of superovulated rats with I.P. injection
385 of NK3R agonist, presumably due to a higher level of follicle maturation/ovulation (Loffler et
386 al., 2004). [This idea is also in line with the previous reports](#) in sea squirt (*Ciona intestinalis*,
387 a well-documented model for protochordate), in which the *Ciona* versions of tachykinins and

388 NKBs are both expressed in the gonad (Satake et al., 2004) and in vitro treatment of Ciona
389 oocytes with Ciona tachykinins can advance the process of oocyte maturation from
390 vitellogenic phase to post-vitellogenic stages (Aoyama et al., 2008). Although the details for
391 NKB regulation of oocyte maturation are still unknown, it appears that the role of tachykinins
392 as intraovarian modulators/local regulators is highly conserved in chordate evolution.
393 Within the ovary, detectable levels of NKB and NK3R transcripts can also be identified in
394 luteal endothelial cells and ovarian macrophages and Ca^{2+} mobilization induced by NK3R
395 activation has been noted in in vitro culture of these two cell types (Brylla et al., 2005),
396 suggesting that NKB/NK3R system may also has a role in functional modulation of the
397 microvasculature and immune cell activation in the ovary. It is also worth mentioning that the
398 effects of NKB may not be restricted to the ovary, as notable levels of NKB and NEP
399 transcript expression can be located in cumulus cells encasing the ovulated eggs (Pintado et al.,
400 2003) while NK3R expression has been reported in the oviduct, especially in oviductal
401 epithelial cells (Cejudo Roman et al., 2012). These findings, as a whole, raise the possibility
402 that the NKB/NK3R system may be involved in embryo transfer or early development of
403 blastocyst in the oviduct.

404

405 4.2 *Expression and functions of NKB in the uterus and placenta*

406

407 Besides the gonad, the uterus is also a major site for peripheral expression of NKB/NK3R
408 system as well as other tachykinins (Cejudo Roman et al., 2012; Page et al., 2006; Pennefather
409 et al., 2004b). In human female (Cejudo Roman et al., 2012) and rodents (Pennefather et al.,
410 2004b), NKB and NK3R signals, both at the transcript levels and/or protein levels, can be
411 detected in the endometrium and myometrium of the uterus and co-localized with kisspeptin,
412 GPR54 and NEP expression within the myometrial layer. During pregnancy, NKB is also
413 expressed predominantly in the outer syncytiotrophoblast of the developing placenta (Page et
414 al., 2000) and its expression level can reach 2-3 fold higher than that commonly found in the
415 brain (Page et al., 2006). The anatomical distribution for uterine expression of NKB and
416 NK3R are in accordance to the reported functions of tachykinins as uterotonic agents (Patak
417 et al., 2003; Patak et al., 2000) and local actions of NKB on smooth muscle contraction in
418 myometrial layer (Pennefather et al., 2004b) and vasodilation activity in placental circulation
419 (Brownbill et al., 2003). In mammals, uterine expression of NKB and NK3R can be increased
420 with age (Cintado et al., 2001) and altered with different phases of estrous cycle or pregnancy
421 (Patak et al., 2005; Pennefather et al., 2004b). These modifications are closely associated
422 with the steroid background of the animal as uterine expression of NKB and NK3R is known
423 to be differentially regulated by sex steroids. In OVX mouse, estrogen treatment reduces
424 NKB and NK3R mRNA levels in the uterus mainly by ER α activation, while similar exposure
425 to progesterone can up-regulate NKB but with no effects on NK3R gene expression (Pinto et

426 al., 2009). In intact mouse during diestrous phase (i.e., the period with low estrogen during
427 the estrous cycle), NKB, NK3R and NEP are expressed at high levels in the uterus. During
428 pregnancy, NK1R becomes the dominant form of uterine NKR expressed in the early phase
429 but its predominance is subsequently replaced by NK2R during the later phase of pregnancy
430 (Patak et al., 2005; Pennefather et al., 2004b). In general, uterine expression of NK3R tends
431 to reduce gradually during pregnancy and reach a very low or even undetectable level before
432 parturition (e.g., in human and rat) (Candenas et al., 2001; Patak et al., 2003). Apparently,
433 uterine contraction occurred during various stages of uterine cycle and pregnancy is mediated
434 by different subtypes of NKR, with NK2R as the major form regulating myometrial
435 contraction during the late pregnancy or puriperium period (Patak et al., 2003; Patak et al.,
436 2005).

437

438 Although NKB is expressed at high level in the placenta (Page et al., 2006) and elevated
439 levels of NKB are commonly detected in the plasma in late gestation (Sakamoto et al., 2003),
440 NKB was found to be not essential for normal pregnancy (Topaloglu and Semple, 2011).
441 During pregnancy, both plasma NKB level and uterine NKB gene expression also reduce
442 rapidly to low levels in parallel with the drop in NK3R expression right before parturition
443 (Patak et al., 2005; Sakamoto et al., 2003), implying that the activity level of local
444 NKB/NK3R system is down-regulated in the uterus during purperium period (Page et al.,

2006). Of note, abnormal expression of NKB during late phase of pregnancy can be associated with increasing risk for stress-induced abortion (Pennefather et al., 2004a), preterm labor (Torricelli et al., 2007) and pre-eclampsia (Page et al., 2006). Recent studies also suggest that elevated levels of placental NKB gene expression (Page et al., 2006) and excessive secretion of NKB from the placenta into maternal blood during the third trimester (Page et al., 2000) may be the major cause of pre-eclampsia for both human and animal models (Page, 2010). Although not much is known regarding the placental functions of NKB except for its uterotonic (Pennefather et al., 2004b) and vasodilator actions (Brownbill et al., 2003), it is worth mentioning that high levels of NKB can also be detected in umbilical cord blood, suggesting that placental NKB may enter fetal blood and play a functional role in modulation of feto-placental haemodynamics (Sakamoto et al., 2003; Zulfikaroglu et al., 2007). In human umbilical vein, SP has been previously shown to induce neutrophil adhesion to vascular endothelial cells via NK1R and NK2R activation, which is considered to be critical for subsequent leukocyte recruitment and infiltration during inflammation (Dianzani et al., 2003). Whether placental NKB also plays a role in immunomodulation during fetal development is unclear and still **needs** to be clarified by future investigations. Recently, increased levels of NKB gene expression in myometrial smooth muscle cells (>20 fold of normal tissue) together with elevated expression of NK3R have been reported in human leiomyomas (Canete et al., 2013). These findings raise the possibility that

464 NKB/NK3R dysregulation may be linked with tumorigenesis/cancer formation in the uterus.

465

466 **5. Comparative aspects and emerging story of NKB in fish models**

467

468 5.1 *Comparative aspects of tachykinin evolution: invertebrates vs vertebrates*

469

470 Tachykinins are an ancient group of neuropeptides and their expression can be identified
471 in the brain and gut as well as other tissues of invertebrate species (Satake and Kawada, 2006;
472 Van Loy et al., 2010), e.g., in the endostyle and gonad of sea squirt (Aoyama et al., 2008).
473 Besides the typical functions as neurotransmitters/neuromodulators, endocrine hormones and
474 autocrine/paracrine regulators, tachykinins in invertebrates [can also be found in the salivary](#)
475 [gland in some species, e.g., in mosquito \(Champagne and Ribeiro, 1994\) and octopus \(Kanda](#)
476 [et al., 2003\)](#), and serve as exocrine secretion, e.g., with venom-like activity in octopus (Kanda
477 et al., 2003) or causing vasodilation in the host during blood feeding in mosquito (Beerntsen
478 et al., 1999). The exocrine functions of tachykinins by acting as antimicrobial peptides are
479 also suspected in lower vertebrates, as tachykinin expression has been demonstrated in the
480 skin of amphibians (Li et al., 2006) and more recently in fish species (Mi et al., 2010). To
481 date, two groups of tachykinins, invertebrate tachykinins (Inv-TK) and tachykinin-related
482 peptides (TKRP), have been reported in protostomic invertebrates, including [insects](#) (Predel et

483 al., 2005; Siviter et al., 2000), mollusks (Kanda et al., 2003; Kanda et al., 2007), and
484 echiuroid worms (Kawada et al., 1999), and more recently in coelenterates (Anctil, 2009)
485 (Table.1). In representative species of invertebrates, cognate receptors with differential
486 selectivity for Inv-TK and TKRP respectively have been identified (Satake et al., 2013;
487 Satake et al., 2003) and found to be functionally linked with Ca^{2+} signaling (Torfs et al.,
488 2002a; Torfs et al., 2002b), IP_3 production (Torfs et al., 2000) and cAMP production (Poels et
489 al., 2005) similar to that of mammalian NKRs (see introduction for details).

490

491 In general, TKRPs are expressed mainly in the brain, nervous tissue and various gut
492 regions of invertebrates (Satake and Kawada, 2006), and interestingly, their mature peptides
493 exist as “tandem repeats” in their respective precursor proteins, e.g., up to 13 copies in the
494 case of cockroach TKRP (Predel et al., 2005). Although the N-terminal of TKRPs tends to
495 be highly variable, their C-terminal end all share a well-conserved “FXGXR” motif, which is
496 structurally homologous to the signature domain “FXGLM” found in vertebrate tachykinins
497 (Table.2). The expression of Inv-TKs, however, is restricted to the salivary gland, and unlike
498 the case of TKRPs, only a single copy of mature peptide can be mapped within their precursor
499 sequences (Satake and Kawada, 2006; Van Loy et al., 2010). At variance to TKRPs with a
500 “FXGXR” motif, the signature motif “FXGLM” of vertebrate tachykinins, which was
501 assumed to take its first appearance in the protochordate (e.g., *Ciona* Ci-TK-I & -II) (Satake et

502 al., 2004), can also be found in the C-terminal of Inv-TKs (Satake et al., 2013). Given that (i)
503 the tissue expression of TKRPs is more consistent with the role of brain/gut peptides and (ii)
504 TKRP but not Inv-TK can induce muscle contraction in invertebrate gut preparation, TKRP is
505 considered to be the functional equivalence of vertebrate tachykinins while Inv-TK is
506 believed to be a form of exocrine secretion for different biological functions (e.g., in mosquito
507 & octopus) (Satake and Kawada, 2006). Of note, almost all of the TKRP mature peptides
508 reported are flanked by two dibasic endoproteolytic sites (“KR/KK/RR” & “GKK/GKR/GRR”)
509 in their precursors (Table.1), a phenomenon that is comparable with vertebrate tachykinins
510 (Table.2) and presumably plays a key role in the release of mature peptides. In contrast, the
511 flanking with dibasic protein cleavage sites is not apparent in Inv-TKs and the
512 post-translational processing leading to the release of Inv-TK mature peptide is still unclear.
513 Since the “GKK/GKR/GRR” downstream flanking motif of TKRP mature peptides is also a
514 target site for protein processing by peptidyl- glycine α -amidating monooxygenase (Martinez
515 and Treston, 1996), it is logical to assume that TKRP mature peptides are released as
516 C-terminal α amidated polypeptides as in the case of vertebrate tachykinins, which is in
517 agreement with the idea that α amidation in tachykinins is essential for receptor binding and
518 activation (Almeida et al., 2004).

519

520 Although different models of tachykinin evolution have been proposed based on sequence

521 analysis and structural organization of Inv-TKs and TKRPs (Satake et al., 2013; Satake et al.,
522 2003), the picture starts to emerge with the recent identification of tachykinins in sea anemone,
523 a representative of diploblastic coelenterates. Recently, data mining with the genome database
524 of starlet sea anemone (*Nematostella vectensis*, Putnam et al., 2007) has revealed the presence
525 of two cnidarian tachykinins, namely Nv-TK-I and Nv-TK-II (Anctil, 2009). Apparently,
526 Nv-TK-I is a member of TKRPs with the C-terminal signature motif “FXGXR”, but unlike
527 the typical organization of TKRP with multiple copies of mature peptides, only a single copy
528 of TKRP mature peptide could be located in the C-terminal region of the cnidarian precursor
529 (Table.1), which is highly comparable to the structural organization of Inv-TK. For the other
530 member of cnidarian tachykinins, Nv-TK-II with 16 identical repeats of the so-called
531 “incomplete TKRP consensus” was once believed to be an evidence for the presence of a
532 “typical TKRP” in the coelenterate ancestor (Satake et al., 2013), but this incomplete
533 consensus shares little sequence homology with other invertebrate tachykinins and does not
534 contain either the “FXGXR” or “FXGLM” signature motif. Our blast search in NCBI
535 protein database with Nv-TK-II full-length a.a. sequence also found that Nv-TK-II was not a
536 cnidarian tachykinin but rather a member of collagen triple helix repeat (THR) family proteins
537 (Supplemental Data, Fig.S1). Phylogenetic analysis based on nucleotide sequences further
538 confirms that Nv-TK-II could be clustered within the clade of THR-containing collagens but
539 not TKRP or Inv-TK families (Fig.2).

540

541 Based on the information available, we postulate that a TKRP similar to Nv-TK-I with a
542 single copy of mature peptide might serve as the ancestral gene for tachykinin evolution
543 (Fig.3). During the evolution of invertebrates, multiple events of segmental duplication of
544 the gene fragment covering the mature peptide might have occurred after the cnidarian
545 ancestor, which might contribute to the formation of TKRP lineage with multiple copies of
546 mature peptides with the “FXGXR” sequence in protostomes including echiuroid worms,
547 mollusks and insect (Satake et al., 2013). Along the way, single a.a. mutation leading to M
548 for R substitution in the “FXGXR” motif and subsequent selection for L residue at position 2
549 from the C-terminal in the ancestral TKRP with a single copy of mature peptide might lead to
550 the appearance of Inv-TK lineage with the “FXGLM” motif, e.g., in mosquito and octopus
551 (Satake et al., 2003). During the evolution of deuterostomes from the invertebrate ancestors,
552 which are believed to have happened 700 million years ago (Grimmelikhuijzen and Hauser,
553 2012), a tandem duplication of the gene fragment covering the Inv-TK mature peptide
554 followed by a.a. mutations/fragment insertion to generate sequence diversity in the N-terminal
555 of mature peptides might have occurred in the deuterostome ancestor, which gave rise to the
556 bipartite organization of tachykinin precursors found in protochordates (e.g., Ciona Ci-TK).
557 During vertebrate evolution, the tachykinin family was further **expanded** into TAC1 encoding
558 SP and NKA, TAC2/3 encoding NKB (with loss of one of the duplicated mature peptide in

559 reptiles, bird and mammals), and TAC4 encoding HK-1 and EKs, presumably caused by the
560 2R whole-genome duplication happened before the splitting between tetrapods and ray-finned
561 fish (Dehal and Boore, 2005). During the process, neofunctionalization and/or
562 subfunctionalization with concurrent nonfunctionalization (by forming pseudogenes via
563 degenerative mutations) or loss of redundant genes (He and Zhang, 2005) might have
564 occurred and contributed to both the structural and functional divergence of tachykinin gene
565 products found in vertebrates. Since a 3R whole-genome duplication had also occurred
566 during the evolution of ray-finned fish 200-300 million years ago, probably after the
567 branching of bony fish from sturgeons (Moghadam et al., 2011; Yuan et al., 2010), the
568 resulting tetraploidization followed by rediploidization caused a rapid evolution with
569 increased diversity in the fish genome (Ravi and Venkatesh, 2008; Volff, 2005). As a result,
570 additional gene duplication for individual members of the tachykinin family (e.g., TAC3 &
571 TAC4) can also be noted in modern-day bony fish.

572

573 5.2 *Emerging story of TAC3 gene products in fish models*

574

575 The comparative aspects of tachykinins have become even more interesting with the recent
576 identification of the novel gene product NKB-related peptide (NKBRP, also called neurokinin
577 F) encoded by zebrafish TAC3 genes (Biran et al., 2012; Ogawa et al., 2012), which is the

578 structural counterpart of the “missing mature peptide” in the NKB precursors of reptiles, bird
579 and mammals. In bony fish, the bipartite organization of tachykinin precursors similar to
580 that of protochordates (Satake et al., 2004) is well conserved in TAC3, but with gene
581 duplication into TAC3a and TAC3b paralogues, e.g., in zebrafish and Atlantic salmon (Biran
582 et al., 2012; Zhou et al., 2012). In the case of zebrafish, these duplicated genes **are** located in
583 close proximity to or among the genes within the HOX gene clusters (Biran et al., 2012; Zhou
584 et al., 2012) similar to that of TAC3 reported in mammals (Conlon and Larhammar, 2005).
585 Each of them encodes its own version of the 10/11 a.a. NKB and 13 a.a. NKBRP, and except
586 for the NKB encoded by TAC3b with a “FXGLL” motif **which was first described in human**
587 **EKC and EKD (Page et al., 2003)** but not found in other tachykinins (Table 2), these zebrafish
588 TAC3 gene products all carry the C-terminal signature motif “FXGLM” typical of tachykinin
589 family (Biran et al., 2012; Ogawa et al., 2012; Zhou et al., 2012). For nomenclature purpose
590 in this article, NKB and NKBRP encoded by TAC3a are referred to as NKBa and NKBRPa
591 while their counterparts encoded by TAC3b are named NKBb and NKBRPb, respectively.

592

593 Similar to NKB in other vertebrates, the mature peptides of NKBRPa and NKBRPb are
594 flanked by the dibasic cleavage sites “KR” and “GRR” in their respective precursors (Table.2),
595 implying that they can be released as α amidated peptides similar to that of other tachykinins.
596 Data mining of NCBI database also reveals the presence of TAC3a and b genes in goldfish

597 (GenBank no: KF177342 & KF177343) and grass carp (GenBank no: JN105351 & KJ577570;
598 submission from our group). Alignment of the respective mature peptide sequences with that
599 of zebrafish and Atlantic salmon unveils a high level of sequence conservation in cyprinid
600 species (Table.3). In this case, the mature peptides for NKBa, NKBb and NKBRPa,
601 respectively, were found to be identical among zebrafish, goldfish and grass carp. Despite
602 the two “semi-conserved” a.a. substitutions compared to the zebrafish sequence, the NKBRPb
603 mature peptides in goldfish and grass carp also share the same a.a. sequence. Of note, the
604 gene products of TAC3a in cyprinids, NKBa and NKBRPa, are also **highly comparable to that**
605 **of salmon (with only one a.a. substitution for NKBa & two a.a. substitutions for NKBRPa),**
606 while the corresponding sequences of the gene products of TAC3b, NKBb and NKBRPb, tend
607 to be more diverse, especially in the N-terminal region. These observations are consistent
608 with the idea of neofunctionalization of duplicated genes with one retaining its “basic protein
609 sequence” to maintain its original function while the other with structural diversity to explore
610 new functional niches during evolution (Li et al., 2005). Among the two peptide products of
611 TAC3b, NKBb is particularly interesting, as a single a.a. mutation with L for M substitution in
612 the signature domain “FXGLM” has created a new consensus motif “FXGLL”. Similar motif
613 has also been identified in human EKC and EKD (Table.2), despite the fact that the other
614 peptide products of human TAC4, namely EKA and EKB, still have the original “FXGLM”
615 sequence (Page et al., 2003). Although the functional relevance of the new motif “FXGLL”

616 is unclear, a T for M mutation in the signature motif “FXGLM” of NKB is known to cause
617 hypogonadism and infertility in human (Topaloglu et al., 2009) and the L for M mutation in
618 NKBb probably will also have a functional impact on the biological actions of the NKB
619 isoform in fish models.

620

621 In zebrafish, TAC3a and TAC3b transcripts are widely expressed in various tissues with
622 high levels of signals detected in the hypothalamus and ovary (Biran et al., 2012; Zhou et al.,
623 2012). Consistent with the reproductive functions of NKB/NK3R system in mammals, NKBa,
624 NKBRPa and NKBRPb, and to a lower extent for NKBb, were all effective in activating
625 zebrafish NK3R expressed in COS-7 cells (Biran et al., 2012; Zhou et al., 2012) and I.P.
626 injection of NKBa, NKBb and NKBRPa could elevate serum level of LH in zebrafish (Biran
627 et al., 2012). Although estrogen treatment in mammals (e.g., rat) can inhibit gene expression
628 of NKB, NK3R and kisspeptin in hypothalamic ARC, which constitute a key component of
629 steroid negative feedback on GnRH neurons (Gill et al., 2012; Navarro et al., 2011a), similar
630 treatment, however, could up-regulate TAC3a but not TAC3b with parallel rises of NK3R,
631 GnRH and kisspeptin gene expression in the brain of zebrafish (Biran et al., 2012; Servili et
632 al., 2011). These findings suggest that (i) NKB neurons in fish may produce two distinct
633 signals for NK3R activation, namely NKB and NKBRP, and (ii) the NKB/NK3R system for
634 kisspeptin/GnRH regulation in fish may constitute a positive rather than negative feedback by

635 estrogen (e.g., in rodents) on the gonadotropic axis. Since (i) the KNDy neurons in ewes are
636 known to be activated during estrogen positive feedback for LH surge (Merkley et al., 2012)
637 and (ii) positive feedback on kisspeptin expression by estrogen via ER α has been reported in
638 the brain of medaka (Mitani et al., 2010), it raises the possibility that the kisspeptin/GnRH
639 system in fish may be more comparable to that of the sheep model. It is also worth mentioning
640 that duplicated genes for kisspeptin (e.g., Kiss1 & Kiss2) and kisspeptin receptor (e.g., Kiss-R1
641 & Kiss-R2) have been identified in fish species, e.g., zebrafish and medaka (Kitahashi et al.,
642 2009; Ogawa and Parhar, 2013). Each of the Kiss/Kiss-R isoforms is known to have its own
643 distinct pattern of distribution in the brain, e.g., with Kiss1 and Kiss2 separately expressed in
644 NVT and NRL nuclei of the hypothalamus in medaka (Mitani et al., 2010) and with Kiss1/
645 Kiss-R1 located in the habenula and Kiss2/Kiss-R2 in the preoptic area and hypothalamus of
646 zebrafish (Ogawa and Parhar, 2013; Servili et al., 2011). In medaka, the Kiss1 neurons in
647 NVT but not Kiss2 neurons in NRL are responsible for central regulation of reproduction by
648 steroid feedback (Mitani et al., 2010), suggesting that the two kisspeptin isoforms may have
649 different physiological functions in fish model. Using in situ hybridization, a recent study in
650 zebrafish has demonstrated that TAC3a is expressed in neurons within the habenula, preoptic
651 area and hypothalamus while TAC3b expression can be found only in the telencephalon
652 (Ogawa et al., 2012). In the same report, interestingly, TAC3 and kisspeptin signals were
653 located in separate neuronal populations within the habenula and hypothalamus, suggesting

654 that the “KNDy model” in mammals with co-expression of kisspeptin, NKB and Dyn in the
655 same neuronal population may not be applicable to the fish model. Given that (i) KNDy
656 neurons represent only a subpopulation of kisspeptin neurons within the CNS (Lehman et al.,
657 2010), and (ii) kisspeptin neurons with no noticeable levels of NKB and Dyn co-expression
658 are also involved in GnRH regulation, e.g., kisspeptin neurons in AVPV in rat (Dungan et al.,
659 2006) or POA in sheep (Smith et al., 2009), we do not exclude the possibility that functional
660 interactions of NKB and kisspeptin neurons may still play a role in GnRH regulation in
661 zebrafish.

662

663 In mammals, tachykinins are known to have direct effects acting at the pituitary level. For
664 examples, SP produced in the pars tuberalis of the anterior pituitary can stimulate PRL release
665 and serve as an autocrine/paracrine regulator for seasonal changes of PRL secretion in sheep
666 (Skinner et al., 2009). In rat pituitary cells, SP and NKB can induce PRL release via NK1R
667 and NK3R activation, respectively (Henriksen et al., 1995). In the same cell model, treatment
668 with SP also triggers Ca^{2+} mobilization (Mau et al., 1997) and PI turnover (Mau et al., 1990),
669 which probably play a functional role in mediating SP’s actions in the pituitary. In zebrafish,
670 transcript signals for TAC3a, and to a lower extent for TAC3b, not only can be located in the
671 hypothalamus and other brain areas but also in the pituitary with notable levels of NK3R gene
672 expression (Biran et al., 2012; Zhou et al., 2012), suggesting that the TAC3 gene products,

673 namely NKB and NKBRP, may have autocrine/paracrine actions within the fish pituitary. In
674 our recent study in grass carp pituitary cells, basal levels of LH secretion as well as LH β ,
675 FSH β and GtH α transcript expression were not affected by static incubation with grass carp
676 NKBa and NKBRPa (Hu et al., 2014). These results are consistent with a recent study in
677 mouse pituitary gonadotroph L β T2 cells, in which NKB was found to have no effects on both
678 basal as well as GnRH-induced LH β and FSH β gene transcription despite the fact that
679 endogenous expression of NK3R could be detected in the cell line (Mijiddorj et al., 2012).
680 Although the two TAC3a gene products did not have direct effects on LH and FSH regulation
681 at the pituitary level, NKBa and NKBRPa treatment, however, were found to up-regulate PRL
682 and somatolactin (SL) α (SL α) secretion, protein production and transcript expression in carp
683 pituitary cells. Apparently, the stimulatory effects of these two TAC3a gene products on
684 PRL and SL α expression were mediated by pituitary NK2R and NK3R, respectively, via
685 functional coupling with the AC/cAMP/PKA, PLC/IP₃/ PKC and/or Ca²⁺/CaM/CaMK-II
686 signaling pathways (Hu et al., 2014). In fish models, similar to mammals, PRL is involved
687 in a wide range of biological actions ranging from organogenesis (Nguyen et al., 2008),
688 osmoregulation (Sakamoto and McCormick, 2006), immune responses (Harris and Bird, 2000)
689 to reproduction (Whittington and Wilson, 2013). Similar to PRL, SL is also a family
690 member of the GH gene lineage (Forsyth and Wallis, 2002) and known to have pleiotropic
691 functions including background adaption, reproduction, lipid metabolism, acid-base balance

692 and immune cell activation (Kawauchi et al., 2009). To date, two forms of SL, SL α and SL β ,
693 have been identified in separate populations of pituitary cells within the posterior pituitary,
694 e.g., in zebrafish (Zhu et al., 2004) and grass carp (Jiang et al., 2008), and suspected to have
695 overlapping and yet distinct functions (Zhu et al., 2007). The demonstration of TAC3a gene
696 products, NKBa and NKBRPa, as novel regulators for PRL and SL α secretion and gene
697 expression in the carp pituitary for sure will add onto the functional complexity of tachykinins
698 in fish models. Whether the TAC3b gene products, namely NKBb and NKBRPb, also have
699 regulatory functions at the pituitary level is unclear and still awaits for further investigations.

700

701 **6. Concluding remarks and future perspectives**

702

703 In the past 5 years, significant progress has been made in the “KNDy neuron” model for
704 GnRH regulation, control of LH pulsatility as well as the mechanisms for estrogen negative
705 feedback in mammals. However, the model is not yet complete and there are still areas with
706 questions for future exploration. For examples, the feedback based on bilateral/autosynaptic
707 innervation of KNDy neurons is supported by the functional data of NKB induction via NK3R
708 and Dyn inhibition via KOR on both basal and/or kisspeptin-induced GnRH neuron activity/
709 LH secretion (Goodman et al., 2013b; Navarro, 2012; Rance et al., 2010), but it is still unclear
710 if local release of NKB within the ARC can also affect Dyn expression in KNDy neurons or

711 vice versa. Besides, the previous studies on KNDy neurons were focused mainly on LH
712 release (e.g., rat) and LH pulsatility (e.g., sheep) and not much is known regarding the effects
713 on FSH secretion/gene expression, not to mention a general lack of information on pituitary
714 actions of NKB despite the fact that KNDy nerve fibers can be found in the median eminence
715 with portal blood vasculature linking to the pituitary (e.g., rat) (Ciofi et al., 2006; Krajewski et
716 al., 2005). In other tissues/cell models, tachykinin degradation via NEP and NEP-2 also play
717 a role in fine tuning the local actions of NKB, e.g., in human uterus (Patak et al., 2003) or
718 mature spermatozoa (Pinto et al., 2010). However, the possible involvement of NEP/NEP-2
719 in the reproductive functions of KNDy neurons has not been examined in mammals.

720

721 Of note, KNDy neurons in the ARC are believed to be the “driving force” for GnRH pulse
722 generator (Navarro, 2013), but recent studies may suggest the otherwise. Unlike the case of
723 human, mice with loss-of-function mutations in NK3R **have relatively mild reproductive**
724 **phenotypes during juvenile phase (e.g., smaller testes in male and reduced uterine weight in**
725 **female) but with normal puberty onset, reproductive cyclicity and fertility in adulthood** (Yang
726 et al., 2012). In human patients with NKB or NK3R mutations, continuous infusion of
727 kisspeptin was found to restore LH pulsatility (Young et al., 2013), suggesting that **the pace-**
728 **maker activity of GnRH neurons is dependent on kisspeptin and can operate without a NKB/**
729 **NK3R system.** These findings raise the possibility that kisspeptin output from KNDy neurons

730 by mechanisms other than NKB/NK3R activation and/or kisspeptin released from “non-KNDy”
731 neurons from other brain areas may also contribute to the regulation of GnRH pulsatility. The
732 details of the mechanisms are still an area of active research and more information is expected
733 to come out in the near future.

734

735 The studies of NKB become even more interesting with the recent discovery of the novel
736 tachykinin member NKBRP in fish models (Biran et al., 2012; Ogawa et al., 2012).
737 Although the biological functions of NKBRP are still at the early phase of investigation, the
738 initial studies in zebrafish have clearly shown that NKBRPa was highly potent in activating
739 both human and zebrafish NK3R expressed in COS-7 cells (Biran et al., 2012). Given that
740 the structure-activity relationship has been a major focus of tachykinin research, mainly for
741 rational design of therapeutic tools for human diseases (Almeida et al., 2004), the clinical
742 implication of NKBRP for future NKR agonist/antagonist development cannot be excluded.
743 In zebrafish, the recent demonstration of NKB and kisspeptin expression in separate neuronal
744 populations within the hypothalamus is intriguing. Whether NKB and NKBRP also play a
745 role in regulating kisspeptin/GnRH expression in brain areas relevant to reproductive function
746 in fish model or have novel functions at the pituitary level as in the case of grass carp for sure
747 are important questions waiting to be clarified by future investigations.

748

749

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751

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757 initial studies.

758

759 **Legends**

760

761 Fig.1. “KNDy neuron” model for GnRH regulation and steroid feedback in mammals. In
762 the hypothalamus, KNDy neurons located within the arcuate nuclei via bilateral/autosynaptic
763 innervation can trigger kisspeptin (Kiss) secretion through type 3 neurokinin receptor (NK3R)
764 activation caused by local release of neurokinin B (NKB). Kiss output from KNDy neurons
765 through activation of its cognate receptor GPR54 not only stimulates GnRH neurons with cell
766 bodies located in the preoptic area, but also trigger GnRH secretion into portal blood through
767 direct innervation of GnRH nerve terminals located in the median eminence. Besides the
768 stimulatory action of NKB, dynorphin A (Dyn) secretion from KNDy neurons, presumably via
769 mediation of a yet unidentified interneuron with κ -type opioid receptor (KOR) expression,
770 can exert a negative feedback to inhibit both basal as well as NKB-induced Kiss release. The
771 functional interplay of the NKB/NK3R system and Dyn/KOR system in the arcuate nuclei can
772 regulate GnRH secretion into hypophysial portal blood, which then controls the pulsatility of
773 LH release from the pituitary into systemic circulation. The neuronal circuitry in the arcuate
774 nuclei with KNDy neurons as a major component also serves as the major target for negative
775 feedback by sex steroids including estrogen and progesterone. Other than the KNDy neurons,
776 GnRH neurons within the preoptic area also receive the signal input of Kiss neurons located
777 in the anteroventral periventricular nuclei, which are believed to be the target site within the

778 hypothalamus responsible for positive feedback of estrogen observed during the preovulatory
779 period (e.g., in rodents).

780

781 Fig.2. Phylogenetic analysis of sea anemone Nv-TK-II nucleotide sequence. Using the
782 nucleotide sequences of collagen triple helix repeat (THR) proteins, invertebrate tachykinins
783 (Inv-TK) and tachykinin-related peptides (TKRP) of various species, rooted analysis using
784 maximum parsimony method with MEGA 5.0 (A) and unrooted analysis using neighbor-
785 joining method with PHYLIP and TreeView program (B) were performed with the Nv-TK-II
786 sequence. The numbers indicated at the branch points of the dendrogram for rooted analysis
787 are the percentage based on 1000 bootstraps, whereas the scale bar shown on the side of the
788 guide tree for unrooted analysis represents the evolution distance. The nucleotide sequences
789 used for phylogenetic analysis were downloaded from the GenBank. [Sea anemone Nv-TK-II,
790 Nv88765; *Macaca mulatta* collagen THR protein, XP2798555; *Conexibacter woesei* collagen
791 THR protein, YP3396840; *Clostridium difficile* collagen THR protein, WP21398144; Octopus
792 Inv-TK OctTK-I, AB85916; Octopus Inv-TK OctTK-II, AB85916; Octopus TKRP, AB96700;
793 cockroach TKRP, AY766011; Beetle TKRP, XP975364; Echiuroid worm TKRP, AB19537;
794 Fruitfly TKRP, NM141884]

795

796 Fig.3. Schematic presentation on the proposed model for tachykinin evolution. In this

797 model, the “ancestral invertebrate tachykinin (Inv-TK)” with structure similar to sea anemone
798 Nv-TK-I (with a single copy of mature peptide with FXGXR as signature motif) underwent
799 multiple cycles of segmental gene duplication covering the region with mature peptide and
800 resulted in the appearance of tachykinin-related peptides (TKRPs) in the protostome lineage
801 with multiple copies of mature peptides carrying FXGXR as signature motif. Meanwhile, the
802 structural organization of ancestral Inv-TK (with a single copy of mature peptide) was still
803 maintained in some invertebrates but with mutation of FXGXR to FXGLM as a new signature
804 motif, which led to the appearance of Inv-TKs in protostomes. A single event of segmental
805 gene duplication of the region with the mature peptide might have happened in Inv-TK during
806 the evolution of protochordate, which formed the basis of bipartite organization of tachykinins
807 found in the deuterostome lineage with two tandem repeats of mature peptides with FXGLM
808 as signature motif. Subsequent 2R and/or 3R whole genome duplication occurred during the
809 evolution from fish to mammals further increased the diversity of tachykinin gene family,
810 despite the loss of some duplicated genes in individual vertebrate classes (e.g., TAC1 in fish
811 & TAC4 in amphibian and bird). Of note, segmental loss of the gene fragment covering the
812 mature peptide of NKBRP in fish and amphibians might have occurred in TAC3 gene of the
813 more advanced forms of tetrapods, including the reptiles, bird and mammals.

814

815

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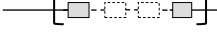
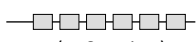
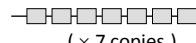
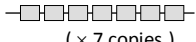
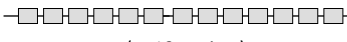
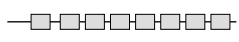




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Table 1.



















Structural organization and mature peptides of cnidarian tachykinins and protostome invertebrate tachykinins (Inv-TKs)/tachykinin-related peptides (TKRPs).

	GenBank No	Mature peptide	Peptide Sequence
Cnidarian Tachykinins			
Sea anemone (<i>Nematostella vectensis</i>)			
(× 1 copy) 	Nv_94714	Nv-TK-I	YQVI F E G V R G K
(× 16 identical repeats) 	Nv_88765	Nv-TK-II	TLQV G R R G R
		Signature motif :	---- F X G X R
Protostome TKRPs			
Fruitfly (<i>Drosophila melanogaster</i>)			
	NM_141884	DTK-1	KRAP T S S E F I G M R G K K
		DTK-2	KKAP L A F V G L R G K K
		DTK-3	KRAP T G F T G M R G K R
		DTK-4	KRAP V N S F V G M R G K K
		DTK-5	KAP N G F L G M R G K K
		DTK-6	K F Q R F A D F N S K F V A V R G K R
Mollusca (<i>Octopus vulgaris</i>)			
	AB096700	Oct-TKRP-I	KKV N P Y S F O G T R G K R
		Oct-TKRP-II	K R L N A N S F M G S R G K R
		Oct-TKRP-III	K R T V S A N A F L G S R G K R
		Oct-TKRP-IV	K K S D A L A F V P T R G R
		Oct-TKRP-V	R R M N S L S F G P P K G K K
		Oct-TKRP-VI	K K Y S P L D E I G S R G K K
		Oct-TKRP-VII	R R A S L H N T F I P S R G K R
Echiuroid (<i>Urechis unicinctus</i>)			
	AB019537	Uru-TK-I	K R L R Q S O F V G A R G K K
		Uru-TK-II	K K A A G M G F F G A R G K K
		Uru-TK-III	K K A A P S G F F G A R G K K
		Uru-TK-IV	K K P R A A Y S G F F G A R G K K
		Uru-TK-V	K K A P S M G F F G A R G K K
		Uru-TK-VI	K K A P H M R F Y G S R G K K
		Uru-TK-VII	K K A P K M G F F G A R G K K
Cockroach (<i>Leucophaea maderae</i>)			
	AY766011	LemTKRP-1	K R A P S G F L G V R G K K
		LemTKRP-2	K R A P A M G F O G V R G K K
		LemTKRP-3	K R G P S M G F H G M R G K K
		LemTKRP-4	K R G P N M G F M G M R G K K
		LemTKRP-5	K R A P S M G F O G M R G K K
		LemTKRP-6	K R A P S M G F O G M R G K K
		LemTKRP-7	K R M G F M G M R G K K
		LemTKRP-8	K R A P A A G F F G M R G K K
		LemTKRP-9	K K V P A S G F F G M R G K K
		LemTKRP-10	K K G P S V G F F A M R G K K
		LemTKRP-11	K K A P S A G F M G M R G K K
		LemTKRP-12	K K A P S G F M G M R G K K
		LemTKRP-13	K K A P S G F L G T R G K K
Beetle (<i>Tribolium castaneum</i>)			
	XP_975364	Tc-TKRP-1	K R A P S G F T G V R G K K
		Tc-TKRP-2	K R A P S G F M G M R G K K
		Tc-TKRP-3	K R A P S G F M G M R G K K
		Tc-TKRP-4	K R A P S G F M G M R G K K
		Tc-TKRP-5	K R A P M G F M G M R G K K
		Tc-TKRP-6	K R A P S G F F G M R G K K
		Tc-TKRP-7	K K M P R Q A G F F G M R G K K
		Tc-TKRP-8	K K Y P Y Q F R G K F V G V R G K K
		Signature motif :	----- F X G X R
Protostome Inv-TKs			
Mosquito (<i>Aedes aegypti</i>)			
(× 1 copy) 	AF108102	Sialokinin I	NTG D K F Y G L M G
(× 1 copy) 	AF108100	Sialokinin II	DTG D K F Y G L M G
Octopus (<i>Octopus vulgaris</i>)			
(× 1 copy) 	AB085916	Oct-TK-I	K P P S S S E F I G L M G R
(× 1 copy) 	AB085917	Oct-TK-II	K P P S S S E F V G L M G R
		Signature motif :	----- F X G L M

The conserved a.a. residues within the signature motif (FXGXR/FXGLM) are underlined in bold type whereas the putative protein cleavage sites flanking the mature peptides (K, KR/KK & GKK/GKR) are labeled in blue. The mature peptide(s) within the respective precursor proteins are presented as grey boxes in the associated structural organization diagrams.

Table 2.

Structural organization and mature peptides of tachykinins in protochordate and vertebrate tachykinins.

	Precursor	Mature Peptide	Peptide Sequence
Protochordate			
<i>Ciona intestinalis</i>	Ci-TK	Ci-TK-I	<u>K</u> RNKRHRV <u>R</u> H <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		Ci-TK-II	<u>K</u> R <u>S</u> I <u>G</u> D <u>Q</u> P <u>S</u> I <u>F</u> N <u>E</u> R <u>A</u> S <u>F</u> <u>T</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
Fish/Teleost			
<i>Danio rerio</i>	TAC1	SP	<u>R</u> K <u>P</u> R <u>P</u> H <u>O</u> <u>F</u> <u>T</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		NKA	<u>K</u> R <u>H</u> K <u>I</u> N <u>S</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
	TAC3a	NKBa	<u>K</u> R <u>E</u> M <u>H</u> D <u>I</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>R</u> <u>R</u>
		NKBRPa	<u>K</u> R <u>Y</u> N <u>D</u> I <u>D</u> <u>Y</u> D <u>S</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>R</u> <u>R</u>
	TAC3b	NKBb	<u>R</u> P <u>N</u> M <u>N</u> D <u>I</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>L</u> <u>G</u> <u>R</u> <u>R</u>
		NKBRPb	<u>K</u> R <u>Y</u> D <u>D</u> I <u>D</u> <u>Y</u> D <u>S</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>R</u> <u>R</u>
	TAC4a	EKA1	<u>K</u> R <u>S</u> K <u>S</u> Q <u>H</u> <u>F</u> <u>H</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>S</u> <u>S</u>
		EKB1	<u>R</u> R <u>N</u> K <u>G</u> E <u>I</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>R</u> <u>R</u>
TAC4b	EKA2	<u>K</u> R <u>S</u> K <u>S</u> H <u>O</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>	
	EKB2	<u>R</u> R <u>H</u> K <u>G</u> D <u>M</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>	
Amphibian			
<i>Xenopus laevis</i>	TAC1	SP	<u>R</u> K <u>P</u> R <u>P</u> D <u>Q</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		NKA	<u>K</u> R <u>Y</u> K <u>S</u> G <u>S</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
	TAC3	NKB	<u>K</u> R <u>E</u> M <u>N</u> D <u>F</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		NKBRP	<u>K</u> R <u>F</u> <u>Y</u> D <u>D</u> D <u>S</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
Reptile			
<i>Alligator sinensis</i>	TAC1	SP	<u>R</u> R <u>P</u> R <u>P</u> Q <u>Q</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		NKA	<u>K</u> R <u>H</u> K <u>T</u> D <u>S</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
	TAC3	NKB	<u>K</u> R <u>D</u> M <u>H</u> D <u>F</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		EKA	<u>K</u> R <u>G</u> K <u>F</u> Q <u>H</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
	EKB	<u>K</u> R <u>A</u> S <u>G</u> D <u>Q</u> G <u>E</u> M <u>F</u> <u>T</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>R</u> <u>R</u>	
Bird			
<i>Gallus gallus</i>	TAC1	SP	<u>R</u> R <u>P</u> R <u>P</u> Q <u>Q</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		NKA	<u>K</u> R <u>H</u> K <u>T</u> D <u>S</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
	TAC3	NKB	<u>K</u> R <u>D</u> M <u>H</u> D <u>F</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
			
Mammals			
<i>Homo sapiens</i>	TAC1	SP	<u>R</u> R <u>P</u> K <u>P</u> Q <u>Q</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		NKA	<u>K</u> R <u>H</u> K <u>T</u> D <u>S</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
	TAC3	NKB	<u>K</u> R <u>D</u> M <u>H</u> D <u>F</u> <u>F</u> <u>V</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
		EKA/B	<u>K</u> T <u>G</u> K <u>A</u> S <u>Q</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
	EKC	<u>R</u> R <u>K</u> K <u>A</u> Y <u>Q</u> L <u>E</u> H <u>T</u> <u>F</u> <u>Q</u> <u>G</u> <u>L</u> <u>L</u> <u>G</u> <u>K</u> <u>R</u>	
	EKD	<u>K</u> R <u>V</u> G <u>A</u> Y <u>Q</u> L <u>E</u> H <u>T</u> <u>F</u> <u>Q</u> <u>G</u> <u>L</u> <u>L</u> <u>G</u> <u>K</u> <u>R</u>	
<i>Mus musculus</i>	TAC4	Hemokinin	<u>K</u> R <u>S</u> R <u>T</u> R <u>Q</u> <u>F</u> <u>F</u> <u>G</u> <u>L</u> <u>M</u> <u>G</u> <u>K</u> <u>R</u>
			
		Signature motif :	----- <u>F</u> <u>X</u> <u>G</u> <u>L</u> <u>M</u>

The conserved a.a. residues within the signature motif (FXGLM) are underlined in bold type whereas the putative protein cleavage sites flanking the mature peptides (R/K, KR/RK/RR & GKK/GKR) are labeled in blue. The mature peptide(s) within the respective precursor proteins are presented as a grey box for Ci-TK-I, grey diamond for Ci-TK-II, black box for SP, white diamond for NKA, white hexagon for NKB, grey box for NKBRP, grey oval box for EKA/B and white oval for EKC/D respectively in the associated structural organization diagrams. Except for TAC3 (with a single copy of mature peptide), other members of protochordate/vertebrate tachykinins have a “bipartite” organization encoding two copies of mature peptides. [GenBank accession numbers of tachykinins for various species have been omitted for simplicity.]

Table 3.

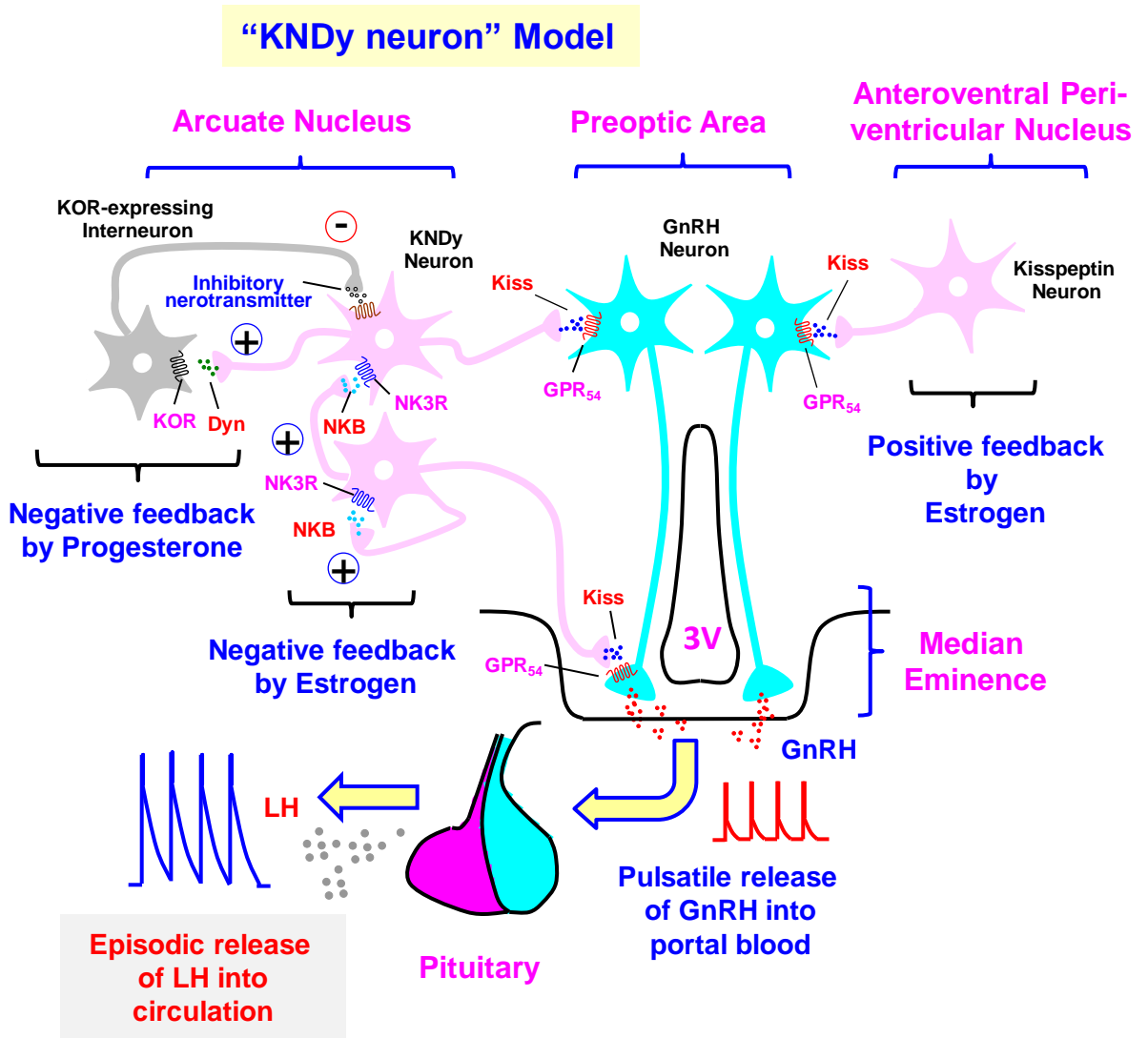
Mature peptides of TAC3a and 3b in bony fish.

A	Teleost TAC3a	
	NKBRPa	NKBa
Zebrafish	KRYNDIDYDS <u>FVGLM</u> GRR	KREMHD <u>I</u> <u>FVGLM</u> GRR
Goldfish	KRYNDIDYDS <u>FVGLM</u> GRR	KREMHD <u>I</u> <u>FVGLM</u> GRR
Grass carp	KRYNDIDYDS <u>FVGLM</u> GRR	KREMHD <u>I</u> <u>FVGLM</u> GRR
Salmon	KRYND <u>L</u> DYDS <u>FVGLM</u> GRR	KREMD <u>D</u> <u>V</u> <u>FVGLM</u> GRR
Signature motif :	----- <u>FVGLM</u>	----- <u>FVGLM</u>

B	Teleost TAC3b	
	NKBRPb	NKBb
Zebrafish	KRY <u>D</u> DIDYDS <u>FVGLM</u> GRR	RPNMND <u>I</u> <u>FVGL</u> <u>L</u> GRR
Goldfish	KRYNDIDYDS <u>F</u> <u>I</u> <u>GLM</u> GRR	RPNMND <u>I</u> <u>FVGL</u> <u>L</u> GRR
Grass carp	KRYNDIDYDS <u>F</u> <u>I</u> <u>GLM</u> GRR	RPNMND <u>I</u> <u>FVGL</u> <u>L</u> GRR
Salmon	KRYRD <u>I</u> <u>H</u> <u>D</u> <u>D</u> <u>T</u> <u>FVGLM</u> GRR	RR <u>S</u> <u>K</u> <u>I</u> <u>R</u> <u>D</u> <u>M</u> <u>D</u> <u>D</u> <u>V</u> <u>FVGL</u> <u>L</u> GRR
Signature motif :	----- <u>FXGLM</u>	----- <u>FVGL</u> <u>L</u>

The conserved a.a. residues within the signature motif (FVGLM/FXGLM/FVGLL) are underlined in bold type while the protein cleavage sites flanking the mature peptides (R/KR & GRR) are labeled in blue. The a.a. substitutions in zebrafish and salmon mature peptides compared with the corresponding sequences in goldfish and grass carp are labeled in red. The M to L mutation in the signature motif of NKBb are highlighted in pink. [GenBank numbers: Zebrafish TAC3a (JN392856) & TAC3b (JN392857); Goldfish TAC3a (KF177342) & TAC3b (KF177343); Grass carp TAC3a (JN105351) & TAC3b (KJ577570); and Atlantic salmon TAC3a (BK008102) & TAC3b (BK008103)]

- Fig.1 -



- Fig. 2 -

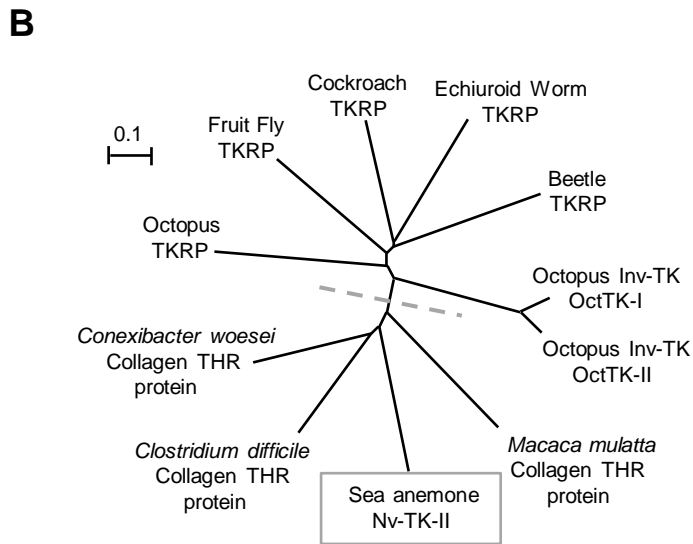
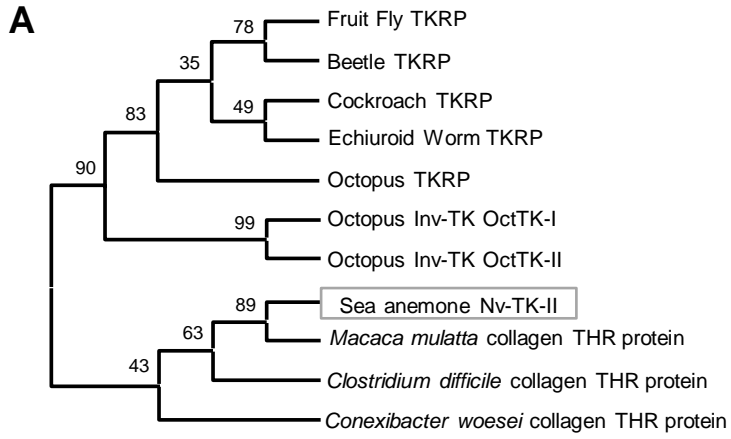
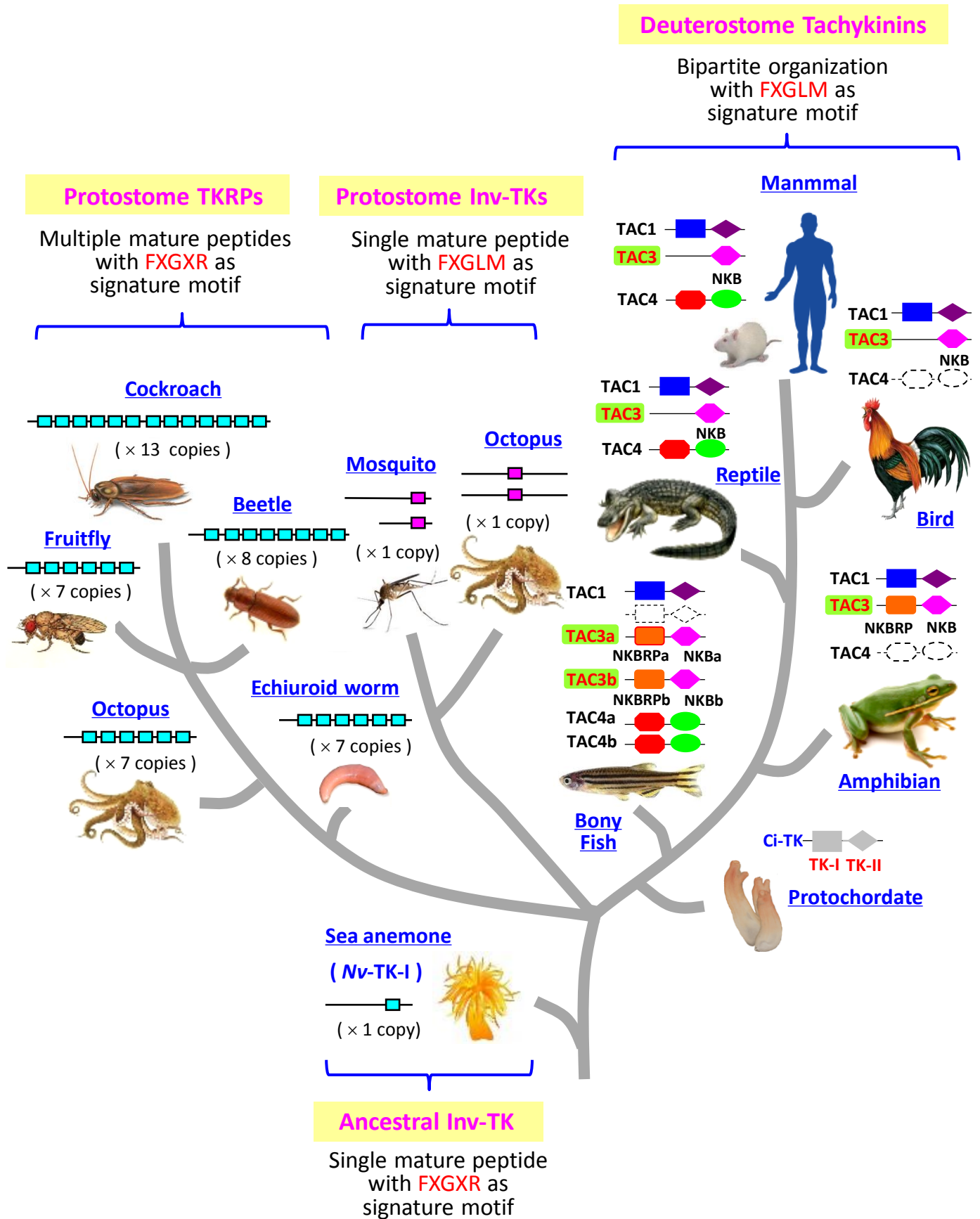


Figure.3

- Fig. 3 -



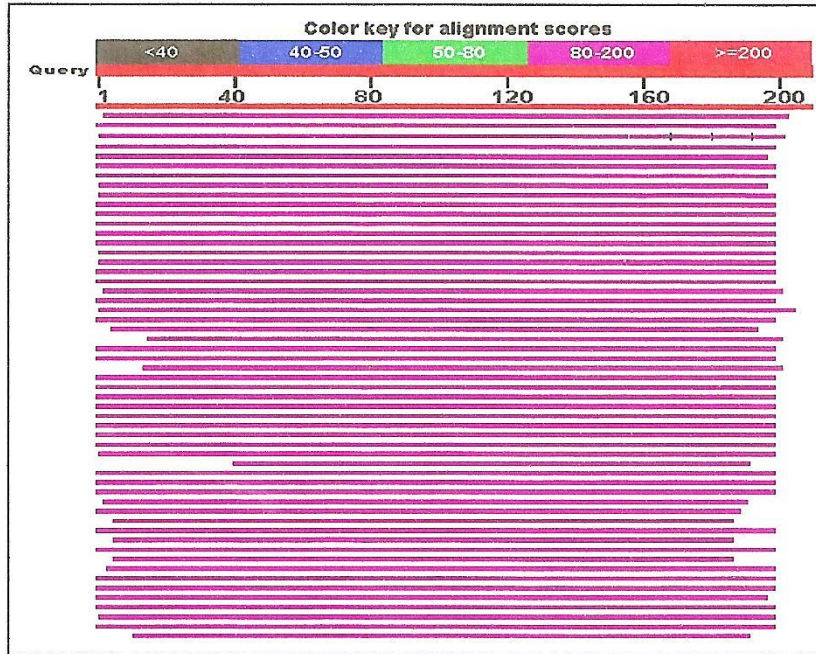
- Supplemental Fig. S1 -

Graphic Summary

Putative conserved domains have been detected, click on the image below for detailed results.



Distribution of 200 Blast Hits on the Query Sequence



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident %	Accession number
Predicted protein [Nematostella vectensis] >gb ED046730.1 Predicted protein [Nematostella vectensis]	330	320	100%	5e-112	100%	XP_001638793.1
PREDICTED: collagen-like protein 1-like [Macaca mulatta] collagen triple helix repeat family protein, partial [Clostridium difficile] >gb EDG66479.1 collagen triple helix repeat family Protein, partial [Clostridium difficile DA00160]	107 106	727 1606	95% 94%	2e-24 3e-24	28% 32%	XP_0027988555.1 WP_021398144.1
Triple helix repeat-containing collagen [Bacillus weihenstephanensis KBAB4] >ref WP_011181891.1 Hypothetical protein [Bacillus weihenstephanensis] >gb ABY44650.1 Collagen triple helix repeat [Bacillus weihenstephanensis KBAB4]	106	1617	95%	6e-23	40%	YP_001646278.1
Hypothetical protein BT92727_4458 [Bacillus thuringiensis Serovar knokukian str. 97-27] >ref WP_011181891.1 Hypothetical protein [Bacillus thuringiensis] >gb AAT63576.1 Collagen-like protein [Bacillus thuringiensis serovar konkukian str. 97-27]	102	8942	94%	2e-21	33%	YP_038772.1
Hypothetical protein [Bacillus cereus] >gb EDX58140.1 collagen triple helix repeat domain protein [Bacillus cereus W]	100	5503	94%	7e-21	33%	WP_001982587.1
Collagen triple helix repeat domain protein [Bacillus cereus AH820] >ref WP_015945709.1 hypothetical protein [Bacillus cereus] >gb ACK88703.1 collagen triple helix repeat domain Protein [Bacillus cereus AH820]	96.7	10493	94%	2e-19	33%	YP_002453790.1

Description	Max score	Total score	Query cover	E value	Ident %	Accession number
Collagen triple helix repeat family protein [Clostridium difficile] >gb EQF27561.1 collagen triple helix repeated Family protein [Clostridium difficile CD160]	96.3	2401	97%	2e-19	32%	WP 021382900.1
Hypothetical protein BCZK4476 [Bacillus cereus E33L] >ref WP_011199027.1 hypothetical protein [Bacillus cereus] >gb AAU15795.1 collagen triple helix repeat family Protein [Bacillus cereus E33L]	95.9	5520	94%	3e-19	33%	YP 086053.1
Collagen triple helix repeat family protein, partial [Clostridium difficile] >gb EQF42316.1 collagen triple helix repeat family Protein, partial [Clostridium difficile CD169]	93.2	277	94%	3e-19	33%	WP 021387037.1
Collagen triple helix repeat family protein, partial [Clostridium difficile] >gb EQF71428.1 collagen triple helix repeat family Protein, partial [Clostridium difficile CD201]	92.4	737	94%	8e-19	32%	WP 021389671.1
Collagen triple helix repeat family protein, partial [Clostridium difficile] >gb ERM51839.1 collagen triple helix repeat family Protein, partial [Clostridium difficile P68]	91.3	442	94%	2e-18	32%	WP 022583078.1
Hypothetical protein [Bacillus cereus] >gb EDX66846.1 collagen triple helix repeat domain protein [Bacillus cereus NVH0597-99]	93.6	3703	96%	2e-18	33%	WP 001991059.1
Hypothetical protein [Bacillus thuringiensis] >gb EEM87400.1 collagen triple helix repeat domain protein [Bacillus thuringiensis serovar pulsiensis BGSC 4AA1]	90.9	710	94%	2e-18	33%	WP 003305569.1
Collagen triple helix repeat family protein, partial [Clostridium difficile] >gb EQH56940.1 collagen triple helix repeat family Protein, partial [Clostridium difficile DA00261]	92.4	2296	97%	3e-18	33%	WP 021405162.1
Collagen triple helix repeat family protein, [Clostridium difficile] >gb EQJ19760.1 collagen triple helix repeat family protein [Clostridium difficile P13] >gb EQJ87425.1 collagen triple helix repeat protein [Clostridium difficile P48] >gb ERM37012.1 collagen triple helix repeat family protein [Clostridium difficile P48]	90.5	1108	94%	1e-17	33%	WP 021405162.1
Collagen triple helix repeat family protein, partial [Clostridium difficile] >gb EQF14205.1 collagen triple helix repeat family Protein, partial [Clostridium difficile CD133]	89.7	1464	94%	2e-17	32%	WP 021387037.1

Fig.S1. Results of BLAST search using the full-length a.a. sequence of sea anemone Nv-TK-II as the query sequence (ID: PRK12678). Except for the positive ID of Nv-TK-II (GenBank no: XP1638793.1) in NCBI database with an alignment score ≥ 200 (shown as horizontal red bar on the top), the alignment scores of the first 50 positive hits are all within the 80-200 range (shown as pink horizontal bars). Among the top hits with highest levels of query coverage, E value and % identity, all of them are members of the collagen triple helix repeat protein family. [Only the first 15 top hits identified by BLAST search were listed with omission of 5 hypothetical proteins with unknown identity in the original list for simplicity.]