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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 Tilte: 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; IP_3, inositol triphosphate; $[Ca^{2+}]i$, intracellular Ca^{2+}; $[Ca^{2+}]e$, extracellular Ca^{2+}; PLD, phospholipase D; CaM, calmodulin; CaMK-II, Ca^{2+}/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, retrochiastmatic area; I.P., intraperitoneal injection; I.C.V., intracerebroventricular injection; OVX, ovarectomy; CL, corpora lutea; NEP, neprilysin; NEP-2, neprilysin-2

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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.

1. Introduction

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

(Lecat et al., 2002; Palanche et al., 2001), PLC-dependent PI hydrolysis (Mizuta et al., 2008; Nakajima et al., 1992), mobilization of IP_3-sensitive $[Ca^{2+}]i$ stores (Mizuta et al., 2008), $[Ca^{2+}]e$ entry via voltage-dependent Ca^{2+} channels (Khawaja and Rogers, 1996; Mau et al., 1997), nNOS-mediated NO and cGMP production (Linden et al., 2000), MAPK activation associated with NKR internalization (Alblas et al., 1996; DeFea et al., 2000), and PLD activation (Torrens et al., 1998) coupled with downstream arachidonic acid release (Garcia et al., 1994). Individual subtypes of NKR, probably via differential interactions with the C-terminal of tachykinins (Satake et al., 2003), are known to exhibit differential selectivity for various members of tachykinins, with NK1R preferring SP (SP > NKA > NKB), NK2R preferring NKA (NKA > NKB > SP) and NK3R preferring NKB (NKB > NKA > SP), respectively (Almeida et al., 2004). With potentials for therapeutic use in human diseases, the structure-activity relationship for ligand/receptor interaction and rational design of agonist/antagonist for different NKR subtypes have been a major focus for tachykinin research (Ganjiwale and Cowsik, 2013).

In recent years, the gene product of TAC3, namely NKB, has emerged as a key regulator for reproductive functions in mammals, especially in the control of GnRH pulsatility within the hypothalamus (Goodman et al., 2013a; Goodman et al., 2013b). The functional role of 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).

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

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

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

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

presence of NK3R mRNA in this cell line together with GnRH secretion with short-term NKB exposure. Interestingly, prolonged treatment with NKB could inhibit GnRH release in GT1-7 cells with a concurrent drop in GnRH gene transcription (Glidewell-Kenney et al., 2013). Whether the inhibitory actions caused by prolonged NK3R activation are also involved in the diversity of GnRH responses reported is not clear, but these biphasic effects of NKB for sure will add to the complexity of "KNDy neuron" model related to species variation, gender difference/steroid background as well as different regulatory targets for NKB within the CNS. It is also worth mentioning that the current studies on the reproductive functions of NKB/ NK3R system are focused mainly on GnRH regulation within the hypothalamus. Given that (i) different NKR subtypes are known to be expressed in the pituitary, e.g., NK1R (Larsen et al., 1992) and NK2R in the rat pituitary (Pisera et al., 2003), and (ii) NKB induction of PRL release and enhancement of TRH-induced PRL gene transcription have been reported in rat pituitary cells (Henriksen et al., 1995) and GH_3 lactotroph cell line (Mijiddorj et al., 2012), respectively, the possibility of LH regulation by NKB via direct action at the pituitary level cannot be excluded.

3. KNDy neurons and steroid negative feedback

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

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

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

The system in other mammals (e.g., in sheep without the AVPV area) is also similar and yet distinct from that of the rodents. Apparently, KNDy neurons in the ARC and its role in estrogen negative feedback during the follicular phase of the ovarian cycle are well conserved whereas species variations have been reported for the kisspeptin neurons mediating estrogen positive feedback (Smith, 2013). For examples, kisspeptin neurons in sheep are also located in the dorsolateral POA close to the region with GnRH neurons, and similar to KNDy neurons in ARC, this neuronal population has been confirmed to have ERα expression (Franceschini et al., 2006). In the same animal model, activation of kisspeptin neurons (as reflected by a rise in c-fos expression) in both ARC and POA can be noted during preovulatory phase (Merkley et al., 2012) or in OVX ewes treated with a high dose of estrogen commonly used to trigger positive feedback during LH surge (Smith et al., 2009). These findings raise the possibility that kisspeptin neurons located in these two brain nuclei are both involved in estrogen positive feedback during preovulatory period in sheep. This idea is also consistent with the findings of a recent study using microimplantation of NK3R agonist in the respective nuclei in ewes to 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 ERβ
305 (Ciofi et al., 1994), the KNDy neurons among the kisspeptin neuronal populations within the
306 ARC are known to express ERα (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 ERα 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 puslatility 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

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

4. Other aspects of NKB in reproductive functions

4.1 *Expression and functions of NKB in the gonad*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In zebrafish, TAC3a and TAC3b transcripts are widely expressed in various tissues with high levels of signals detected in the hypothalamus and ovary (Biran et al., 2012; Zhou et al., 2012). Consistent with the reproductive functions of NKB/NK3R system in mammals, NKBa, NKBRPa and NKBRPb, and to a lower extent for NKBb, were all effective in activating zebrafish NK3R expressed in COS-7 cells (Biran et al., 2012; Zhou et al., 2012) and I.P. injection of NKBa, NKBb and NKBRPa could elevate serum level of LH in zebrafish (Biran et al., 2012). Although estrogen treatment in mammals (e.g., rat) can inhibit gene expression of NKB, NK3R and kisspeptin in hypothalamic ARC, which constitute a key component of steroid negative feedback on GnRH neurons (Gill et al., 2012; Navarro et al., 2011a), similar treatment, however, could up-regulate TAC3a but not TAC3b with parallel rises of NK3R, GnRH and kisspeptin gene expression in the brain of zebrafish (Biran et al., 2012; Servili et al., 2011). These findings suggest that (i) NKB neurons in fish may produce two distinct signals for NK3R activation, namely NKB and NKBRP, and (ii) the NKB/NK3R system for 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 LHβ,
675 FSHβ and GtHα 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 LβT2 cells, in which NKB was found to have no effects on both
678 basal as well as GnRH-induced LHβ and FSHβ 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 (SLα) 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 SLα expression were mediated by pituitary NK2R and NK3R, respectively, via
685 functional coupling with the AC/cAMP/PKA, PLC/IP_3/ PKC and/or Ca^{2+}/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, SLα and SLβ,
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 SLα 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 pusatility 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 pusatility (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

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

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

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Legends

Fig.1. "KNDy neuron" model for GnRH regulation and steroid feedback in mammals. In the hypothalamus, KNDy neurons located within the arcuate nuclei via bilateral/autosynaptic innervation can trigger kisspeptin (Kiss) secretion through type 3 neurokinin receptor (NK3R) activation caused by local release of neurokinin B (NKB). Kiss output from KNDy neurons through activation of its cognate receptor GPR54 not only stimulates GnRH neurons with cell bodies located in the preoptic area, but also trigger GnRH secretion into portal blood through direct innervation of GnRH nerve terminals located in the median eminence. Besides the stimulatory action of NKB, dynorphin A (Dyn) secretion from KNDy neurons, presumably via mediation of a yet unidentified interneuron with κ-type opioid receptor (KOR) expression, can exert a negative feedback to inhibit both basal as well as NKB-induced Kiss release. The functional interplay of the NKB/NK3R system and Dyn/KOR system in the arcuate nuclei can regulate GnRH secretion into hypophysial portal blood, which then controls the pulsatility of LH release from the pituitary into systemic circulation. The neuronal circuitry in the arcuate nuclei with KNDy neurons as a major component also serves as the major target for negative feedback by sex steroids including estrogen and progesterone. Other than the KNDy neurons, GnRH neurons within the preoptic area also receive the signal input of Kiss neurons located 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 dentrogram for rooted analysis
787 are the percentage based on 1000 bootstraps, whereas the scale bar shown on the side of the
788 guidetree 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

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

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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 *(Nematostella vetensis)*			
(× 1 copy)	Nv_94714	*Nv*-TK-I	YQVI**F**E**G**V**R**GK
(× 16 identical repeats)	Nv_88765	*Nv*-TK-II	TL**Q**V**G**R**R**GR
		Signature motif :	----**F**X**G**X**R**
Protostome TKRPs			
Fruitfly *(Drosophila melanogaster)*			
(× 6 copies)	NM_141884	DTK-1	KRAPTSS**F**I**G**M**R**GKK
		DTK-2	KKAPLA**F**V**G**L**R**GKK
		DTK-3	KRAPTG**F**T**G**M**R**GKR
		DTK-4	KRAPVNS**F**V**G**M**R**GKK
		DTK-5	KAPNG**F**L**G**M**R**GKK
		DTK-6	KRQQRFADFNSK**F**V**A**V**R**GKR
Mollusca *(Octopus vulgaris)*			
(× 7 copies)	AB096700	*Oct*-TKRP-I	KKVNPYS**F**Q**G**T**R**GKR
		Oct-TKRP-II	KRLNANS**F**M**G**S**R**GKR
		Oct-TKRP-III	KRTVSANA**F**L**G**S**R**GKR
		Oct-TKRP-IV	KKSDALA**F**V**P**T**R**GR
		Oct-TKRP-V	RRMNSLS**F**G**P**P**K**GKK
		Oct-TKRP-VI	KKYSPLD**F**I**G**S**R**GKK
		Oct-TKRP-VII	RRASLHNT**F**I**P**S**R**GKR
Echiuroid *(Urechis unicinctus)*			
(× 7 copies)	AB019537	*Uru*-TK-I	KRLRQSQ**F**V**G**A**R**GKK
		Uru-TK-II	KKAAGMG**F**F**G**A**R**GKK
		Uru-TK-III	KKAAPSG**F**F**G**A**R**GKK
		Uru-TK-IV	KKPRAAYSG**F**F**G**A**R**GKK
		Uru-TK-V	KKAPSMG**F**F**G**A**R**GKK
		Uru-TK-VI	KKAPHMR**F**Y**G**S**R**GKK
		Uru-TK-VII	KKAPKMG**F**F**G**A**R**GKK
Cockroach *(Leucophaea maderae)*			
(× 13 copies)	AY766011	LemTKRP-1	KRAPSG**F**L**G**V**R**GKK
		LemTKRP-2	KRAPAMG**F**Q**G**V**R**GKK
		LemTKRP-3	KRGPSMG**F**H**G**M**R**GKK
		LemTKRP-4	KRGPNMG**F**M**G**M**R**GKK
		LemTKRP-5	KRAPSMG**F**Q**G**M**R**GKK
		LemTKRP-6	KRAPSMG**F**Q**G**M**R**GKK
		LemTKRP-7	KRMG**F**M**G**M**R**GKK
		LemTKRP-8	KRAPAAG**F**F**G**M**R**GKK
		LemTKRP-9	KKVPASG**F**F**G**M**R**GKK
		LemTKRP-10	KKGPSVG**F**F**A**M**R**GKK
		LemTKRP-11	KKAPSAG**F**M**G**M**R**GKK
		LemTKRP-12	KKAPSG**F**M**G**M**R**GKR
		LemTKRP-13	KKAPSG**F**L**G**T**R**GKK
Beetle *(Tribolium castaneum)*			
(× 8 copies)	XP_975364	*Tc*-TKRP-1	KRAPSG**F**T**G**V**R**GKK
		Tc-TKRP-2	KRAPSG**F**M**G**M**R**GKK
		Tc-TKRP-3	KRAPSG**F**M**G**M**R**GKK
		Tc-TKRP-4	KRAPSG**F**M**G**M**R**GKK
		Tc-TKRP-5	KRAPMG**F**M**G**M**R**GKK
		Tc-TKRP-6	KRAPSG**F**F**G**M**R**GKK
		Tc-TKRP-7	KKMPRQAG**F**F**G**M**R**GKK
		Tc-TKRP-8	KKYPYQFRGK**F**V**G**V**R**GKK
		Signature motif :	--------**F**X**G**X**R**
Protostome Inv-TKs			
Mosquito *(Aedes aegypti)*			
(× 1 copy)	AF108102	Sialokinin I	NTGDK**F**Y**GLM**G
(× 1 copy)	AF108100	Sialokinin II	DTGDK**F**Y**GLM**G
Octopus *(Octopus vulgaris)*			
(× 1 copy)	AB085916	*Oct*-TK-I	KPPSSSE**F**I**GLM**GR
(× 1 copy)	AB085917	*Oct*-TK-II	KPPSSSE**F**V**GLM**GR
		Signature motif :	-------**F**X**GLM**

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			
Ciona intestinalis	Ci-TK 	Ci-TK-I	KRNKRHVRH**F**Y**GLM**GKR
		Ci-TK-II	KRSIGDQPSIFNERAS**F**T**GLM**GKR
Fish/Teleost			
Danio rerio	TAC1 	SP	RKPRPHQ**F**I**GLM**GKR
		NKA	KRHKINS**F**V**GLM**GKR
	TAC3a 	NKBa	KREMHDI**F**V**GLM**GRR
		NKBRPa	KRYNDIDYDS**F**V**GLM**GRR
	TAC3b 	NKBb	RPNMNDI**F**V**GL**LGRR
		NKBRPb	KRYDDIDYDS**F**V**GLM**GRR
	TAC4a 	EKA1	KRSKSQH**F**H**GLM**GSS
		EKB1	RRNKGEI**F**V**GLM**GRR
	TAC4b 	EKA2	KRSKSHQ**F**Y**GLM**GKR
		EKB2	RRHKGDM**F**V**GLM**GKR
Amphibian			
Xenopus laevis	TAC1 	SP	RKPRPDQ**F**Y**GLM**GKR
		NKA	KRYKSGS**F**F**GLM**GKR
	TAC3 	NKB	KREMNDF**F**V**GLM**GKR
		NKBRP	KRFYDDDS**F**V**GLM**GKR
Reptile			
Alligator sinensis	TAC1 	SP	RRPRPQQ**F**F**GLM**GKR
		NKA	KRHKTDS**F**V**GLM**GKR
	TAC3 	NKB	KRDMHDF**F**V**GLM**GKR
	TAC4 	EKA	KRGKFQH**F**Y**GLM**GKR
		EKB	KRASGDQGEM**F**I**GLM**GRR
Bird			
Gallus gallus	TAC1 	SP	RRPRPQQ**F**F**GLM**GKR
		NKA	KRHKTDS**F**V**GLM**GKR
	TAC3 	NKB	KRDMHDF**F**V**GLM**GKR
Mammals			
Homo sapiens	TAC1 	SP	RRPKPQQ**F**F**GLM**GKR
		NKA	KRHKTDS**F**V**GLM**GKR
	TAC3 	NKB	KRDMHD**F**V**GLM**GKR
	TAC4	EKA/B	KTGKASQ**F**F**GLM**GKR
		EKC	RRKKAYQLEHT**F**Q**GL**LGKR
		EKD	KRVGAYQLEHT**F**Q**GL**LGKR
Mus musculus	TAC4	Hemokinin	KRSRTRQ**F**Y**GLM**GKR
		Signature motif :	-------**F**X**GLM**

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

Table 3.

Mature peptides of TAC3a and 3b in bony fish.

A

	Teleost TAC3a	
	NKBRPa	NKBa
Zebrafish	KRYNDIDYDS**FVGLM**GRR	KREMHDI**FVGLM**GRR
Goldfish	KRYNDIDYDS**FVGLM**GRR	KREMHDI**FVGLM**GRR
Grass carp	KRYNDIDYDS**FVGLM**GRR	KREMHDI**FVGLM**GRR
Salmon	KRYNDLDYDS**FVGLM**GRR	KREMDDV**FVGLM**GRR
Signature motif :	--------**FVGLM**	-------**FVGLM**

B

	Teleost TAC3b	
	NKBRPb	NKBb
Zebrafish	KRYDDIDYDS**F**V**GLM**GRR	RPNMNDI**FVGLL**GRR
Goldfish	KRYNDIDYDS**F**I**GLM**GRR	RPNMNDI**FVGLL**GRR
Grass carp	KRYNDIDYDS**F**I**GLM**GRR	RPNMNDI**FVGLL**GRR
Salmon	KRYRDIHDDT**F**V**GLM**GRR	RRSKIRDMDDV**FVGLL**GRR
Signature motif :	--------**F**X**GLM**	---------**FVGLL**

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)]

Figure.1

- Fig.1 -

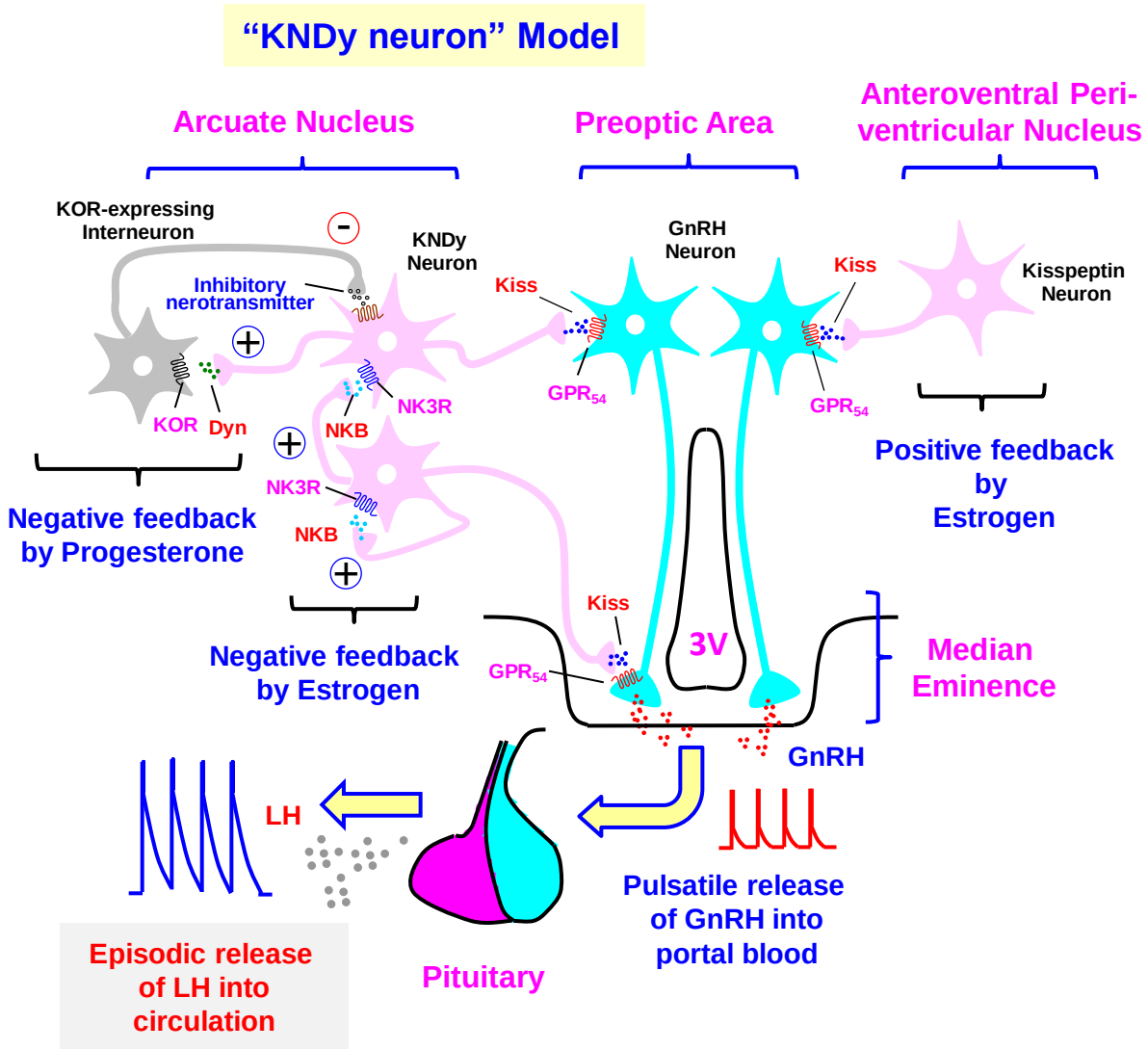


Figure.2

- Fig. 2 -

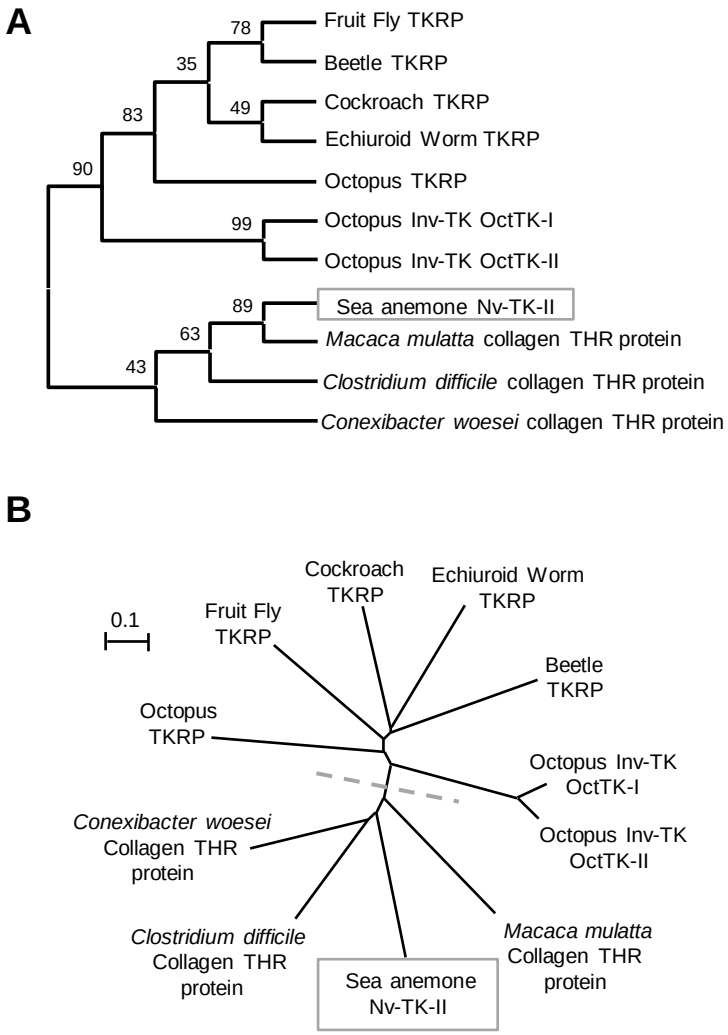
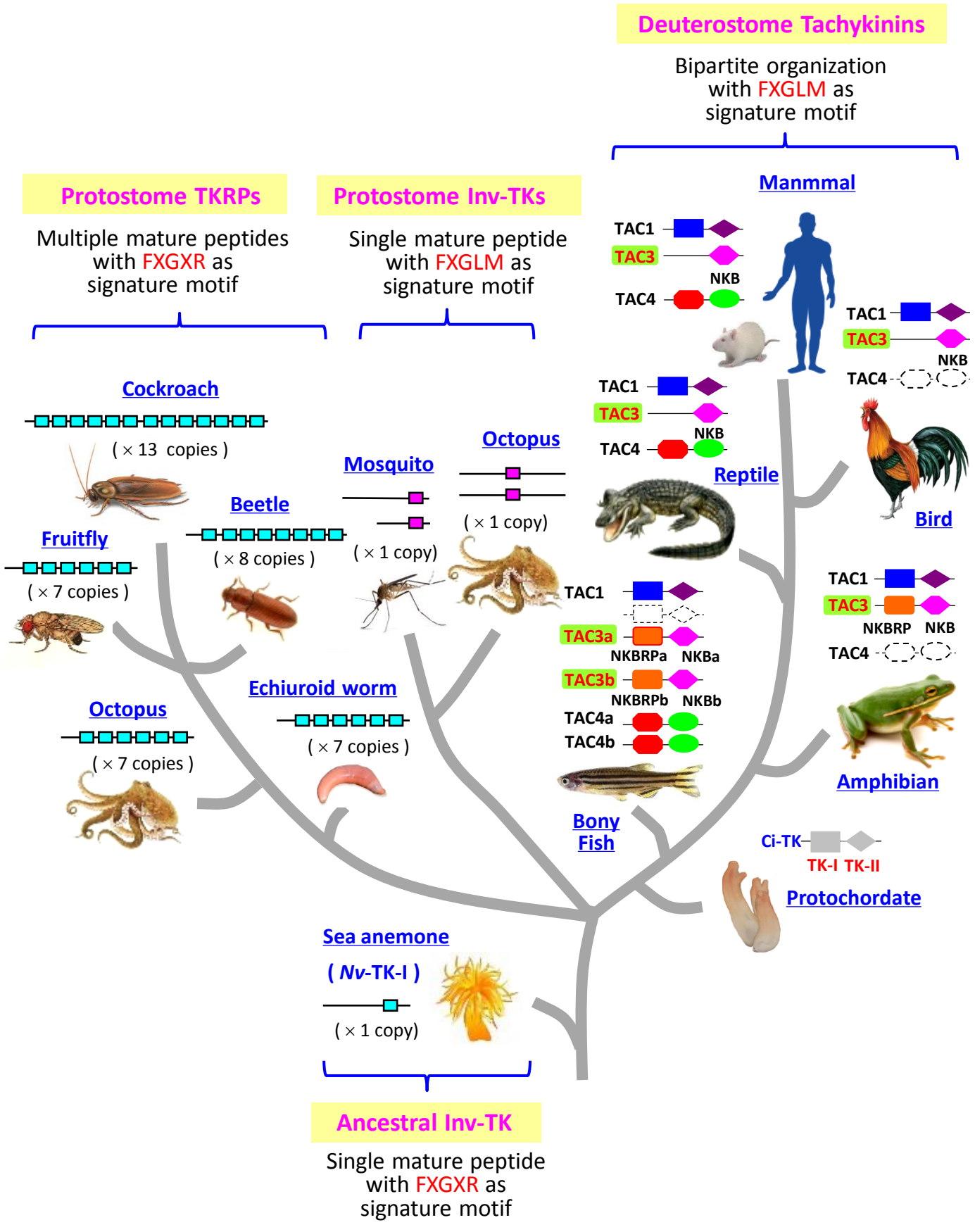


Figure.3

- Fig. 3 -

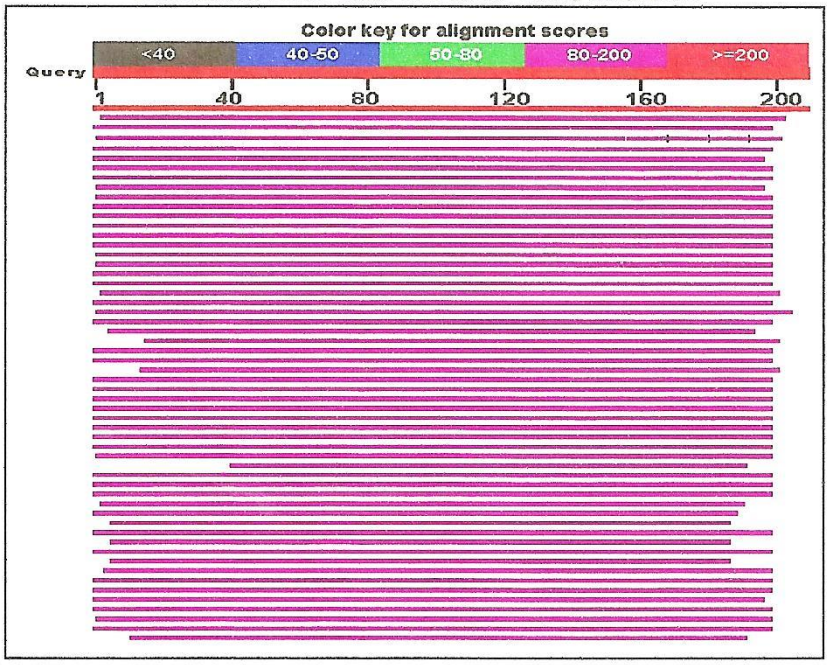


- Supplemental Fig. S1 -

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]	107	727	95%	2e-24	28%	XP 0027988555.1
collagen triple helix repeat family protein, partial [Clostridium difficile] >gb|EDG66479.1| collagen triple helix repeat family Protein, partial [Clostridium difficile DA00160]	106	1606	94%	3e-24	32%	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 heliz 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.]