Retinoid-X Receptor Agonists Increase Thyroid Hormone Competence in Lower Jaw Remodeling of Pre-Metamorphic *Xenopus laevis* tadpoles

Brenda J. Mengeling*, Lara F. Vetter, and J. David Furlow

Department of Neurobiology, Physiology, and Behavior, College of Biological Sciences, University of California, Davis, 1 Shields Ave., Davis, CA 95616

*corresponding author: mengeling@ucdavis.edu

DISCLOSURE STATEMENT: the authors have nothing to disclose

Running Title: RXR agonists increase thyroid hormone competence

Abstract

Thyroid hormone (TH) signaling plays critical roles during vertebrate development, including regulation of skeletal and cartilage growth. TH acts through its receptors (TRs), nuclear hormone receptors (NRs) that heterodimerize with Retinoid-X receptors (RXRs), to regulate gene expression. A defining difference between NR signaling during development compared to in adult tissues, is competence, the ability of the organism to respond to an endocrine signal. Amphibian metamorphosis, especially in *Xenopus laevis*, the African clawed frog, is a well-established in vivo model for studying the mechanisms of TH action during development. Previously, we've used one-week post-fertilization *X. laevis* tadpoles, which are only partially competent to TH, to show that in the tail, which is naturally refractive to exogenous T3 at this stage, RXR agonists increase TH competence, and that RXR antagonism inhibits the TH response. Here, we focused on the jaw that undergoes dramatic TH-mediated remodeling during metamorphosis in order to support new feeding and breathing styles. We used a battery of approaches in one-week-old tadpoles, including quantitative morphology, differential gene expression and whole mount cell proliferation assays, to show that both pharmacologic (bexarotene) and environmental (tributyltin) RXR agonists potentiated TH-induced responses but were inactive in the absence of TH; and the RXR antagonist UVI 3003 inhibited TH action. At this young age, the lower jaw has not developed to the point that T3-induced changes produce an adult-like jaw morphology, and we found that increasing TH competence with RXR agonists did not give us a more natural-metamorphic phenotype, even though Bex and TBT significantly potentiated cellular proliferation and the TH induction of *runx2*, a transcription factor critical for developing cartilage and bone. Prominent targets of RXR-mediated TH potentiation were members of the matrix metalloprotease family, suggesting that RXR potentiation may emphasize pathways responsible for rapid changes during development.

Introduction

An organism's acquired ability to respond both qualitatively and quantitatively to a physiological signal, defined as competence, is distinguished between endocrine signaling during development, which tends to lead to irreversible, organizational effects, from that of healthy adult tissues, which controls the functioning of tissues and organs to maintain homeostasis. Thyroid hormone (TH) action regulates many aspects of vertebrate development including cartilage growth and skeletogenesis (1–4). Over developmental time, the vertebrate organism traverses from low to high TH competence (5). Vertebrate development depends upon appropriate timing and concentrations of TH for good biological outcomes. During human development, adverse outcomes arise from both insufficient and excessive TH (1,6–10). However, analysis of the effects of TH on mammalian development are confounded by maternal effects due to the nature of intrauterine growth. Amphibian metamorphosis, the process through which larval tadpoles develop into adult frogs, overcomes these obstacles; it provides an accessible and dramatic model for direct investigation of the role TH plays during vertebrate development (11–14). Metamorphosis is initiated and maintained through the action of TH (15–18). The African clawed frog, *Xenopus laevis*, provides an accessible and effective laboratory model for assessing the role of TH throughout development, and its metamorphosis has been shown to model the essential perinatal surge in TH signaling in humans (11,14).

In all vertebrates, TH acts through the thyroid hormone receptors (TRs), which are DNA-binding, ligand-regulated transcription factors of the nuclear receptor (NR) superfamily (19,20). THs are identical across all taxa, and the TRs are highly conserved between *X. laevis* and humans (12,13). Two isoforms of TR are expressed from two different genes, TRα and TRβ. In *X. laevis* tadpoles, TRα is expressed before synthesis of THs commences (21), whereas TRβ expression is induced after the nascent thyroid gland begins to synthesize THs through TH binding to TRα; it is a direct target gene of TRs (22,23). 3,3',5-triiodo-L-thyronine (T3) is the TH with the highest affinity for the TRs (24–26). TRs bind DNA and can regulate gene transcription in both the absence and presence of TH. In the most-studied model of TH

action, apo-TRs recruit co-repressor proteins, which close the chromatin environment to the general transcription machinery, causing transcriptional repression. In contrast, T3-bound TRs recruit co-activator proteins, which open the chromatin environment to the general transcription machinery, causing transcriptional activation (19,20). TRs heterodimerize with another NR, the retinoid-X receptors (RXRs) (27). RXRs bind several natural ligands, including 9-cis retinoic acid, and they can dimerize with many different NRs in addition to TRs (28). The TR-RXR heterodimer shows higher affinity for DNA, especially in the presence of T3, than the TR-TR homodimer (29).

Due to the importance of TH signaling for proper development, man-made chemicals that disrupt TR action have the potential to produce adverse outcomes (30). Our initial investigations into disruption of TR function used an integrated TRE-driven Luc reporter in a rat pituitary cell line that endogenously expresses both TRα and TRβ (31,32). In that cell culture system, vitamin A (VA) metabolites were the primary hits for disrupting the TRE-Luc reporter (33). VA metabolites can activate both the retinoic acid receptors (RARs) and the RXRs, depending upon the metabolite (34). RARs, like TRs and RXRs, are members of the NR superfamily. The documented but not mechanistically understood ability of VA metabolites to convert into each other make using them to determine RAR vs RXR function difficult. A pharmaceutical RAR ligand did not affect the TRE-Luc reporter (32), but a pharmaceutical RXR agonist did (33). Since RARs should not bind a TRE, and RXRs heterodimerize with TRs on TREs, these results would appear unsurprising were it not for the fact that, in most adult tissues and cells, RXR ligands are unable to affect the action of the TR-RXR heterodimer (35,36). Pituitary cells, like the reporter cell line, are an exception, wherein RXR ligands do affect the ability of TR to control the hypothalamus-pituitary-thyroid (HPT) axis (37); the biological reasons for this are not understood. In fact, the pharmaceutical RXR agonist used in this study, bexarotene (brand name Targretin, Bex), produces severe hypothyroidism in patients given the drug, which limits its usability as a chemotherapeutic (38–40). However, given the inability of RXR ligands to affect TR function in peripheral tissues, such as the liver—a major site of TR function—the TR-RXR heterodimer is generally considered to be an example of a “non-permissive” RXR heterodimer, meaning that only the ligand for the TR, TH, can induce activation. This is also thought to

"make sense" because TH is an endocrine hormone that tightly regulates several important biological functions like heart rate, and tight, endocrine-type regulation would be complicated if a second hormone or environmental ligand (dietary, etc) were also able to affect TR-RXR action (35).

Tributyltin (TBT) is a pervasive environmental pollutant that was the first described endocrine disruptor when it was discovered that exposure to TBT caused marine gastropods to develop imposex phenotypes, where female gastropods develop male secondary sex characteristics (41,42). TBT was widely used as an antifoulant in marine paints. Mechanistic work determined that TBT functioned through the mollusk RXR, and that treating marine gastropods with either 9-cis retinoic acid or TBT produced the same imposex phenotype (43–45). Biophysical investigations showed that TBT covalently binds to a cysteine residue at the entrance to the RXR ligand-binding pocket, creating an activated conformation in the RXR (46). In the rat pituitary reporter cell line, TBT behaved like Bex, strongly suggesting that it was functioning as an RXR agonist (33,47). These results left open the question as to whether RXR agonists in a developing organism would behave like RXR agonists in most adult tissues (i.e. RXR agonists have no effect on TR action) or like in our pituitary reporter cell line (i.e. RXR agonists could modulate TR function).

Previously, we developed a suite of quantitative assays to assess function and possible disruption of TH action in 1-week post-fertilization (1wk-PF) tadpoles (NF 48) (48). 1wk-PF tadpoles express TRα, but they do not yet have an active thyroid gland; therefore, they are TH negative and are considered pre-competent (21). Addition of T3 to their rearing water activates many metamorphic pathways, but the addition of T3 does not make their TH competence complete. For example, tail resorption, the last step of metamorphosis, is minimal even under supraphysiological doses of T3, because at least in part, the tail expresses high levels of the T3-deactivating enzyme, deiodinase 3 (49). We found that the addition of Bex or TBT to the rearing water of 1wk-PF tadpoles in the presence of T3 significantly potentiated the action of T3 in larval tissues undergoing resorption, including the tail (47,50). In effect TBT/Bex increased T3 competence in the tail to near metamorphic levels. At the transcriptomic level in the tadpole tail we found that TBT acted identically to Bex, solidifying that the mechanism of TBT action on TH function was at

the level of RXR agonism (50). We wondered if the potentiating effects of RXR agonists affected metamorphic phenotypes beyond resorption.

Amphibian metamorphosis affects almost every tissue system and cell fate decision. TH induces the jaw to remodel to facilitate the transition from an herbivorous tadpole to a carnivorous adult frog. During natural metamorphosis, visible jaw morphological changes start at NF 59, which is approximately 45 days post-fertilization (PF) under ideal rearing conditions (51,52). Thomson describes three phases of Meckels cartilage development in the lower jaw (LJ): 1) a lag phase (NF 57-59) with low levels of cell proliferation, 2) a division phase (NF 60-62) of rapid cell division, and 3) a synthesis phase (NF 62-66) wherein the matrix content of the cartilage increases significantly (53,54). Rose showed that tadpoles prior to NF 57 (~41 days PF) respond to the TH but the beak-like morphological changes that result are not seen in a natural metamorphosis (51). Between NF 48 and NF 57 significant, non-TH-induced growth occurs to the cartilages of the lower jaw, and this growth appears to be essential for producing appropriate morphology upon TH administration. Bearing this in mind, we investigated whether RXR ligands were able to potentiate the T3-induced changes that are possible at NF 48, where we have an extant suite of quantitative assays to monitor potential disruption of T3 action (48). We found that both Bex and TBT potentiated T3-induced proliferation, the activation of *runx2*, a transcription factor necessary for maturation of cartilage and bone ossification, and the matrix metalloproteases *mmp11* and *mmp13l*. On the other hand, the RXR antagonist UVI 3003 (UVI) (55) prevented T3-induced morphological changes while not inhibiting proliferation, and it only selectively inhibited gene transcription. In addition, Bex and TBT still potentiated T3 action in the LJ in tadpoles at NF 54, which are considered prometamorphic and fully competent to respond to THs.

Materials and Methods:

Reagents:

3,3',5-triiodo-L-thyronine (T3, T6397-100MG) and tributyltin chloride (TBT, T50202-5G) were purchased from MilleporeSigma (Burlington, MA) and Bexarotene (Bex, 5819/10 and UVI 3003 (UVI, 3303/10) were purchased from Tocris Biosciences (Bio-Techne, Minneapolis, MN). All treatment ligands were dissolved or diluted in dimethyl sulfoxide (DMSO, Thermo Fisher Scientific, Waltham, MA). oLH (ovine luteinizing hormone) was purchased through the National Hormone and Peptide Program (Los Angeles, CA), pregnant mare serum gonadotropin was purchased from Thermo Fisher Scientific, and tricaine methanesulfonate was purchased from Western Medical Supply (Arcadia, CA).

Animal husbandry:

The laboratory has an approved University of California Davis Institutional Animal Care and Use protocol that covers the husbandry and mating of adult *Xenopus laevis* frogs and ligand exposure of larval tadpoles. Wild-type *X. laevis* frogs were mated and embryos cultured as described (Mengeling 2017).

Tadpole precocious metamorphosis morphology assay:

NF 48 (1-week post-fertilization) tadpoles were treated, fixed for photography, and dorsal head photos taken using a Leica DFC3000 G camera on a Leica MZLFIII microscope as described (Mengeling 2016 and 2017). Treatment concentrations, unless otherwise indicated were 10 nM T3, 30 nM Bex, 1 nM TBT, and 600 nM UVI, based upon previous results. The angle of the lower jaw was measured using the FIJI (56) distribution of ImageJ (57). GraphPad Prism 9 (GraphPad Software, La Jolla, CA) was used to generate box and whisker plots, where boxes represent the 25th to 75th percentiles with the bar at the median, and whiskers are maximum and minimum values. For statistical analyses, each animal counted as an individual, and 2 clutches (ten tadpoles/clutch) were assayed independently to control for clutch-to-clutch variability. NF 54 tadpoles were treated as NF 48 animals except that the volume/tadpole of rearing

171 water was increased to 50 ml, and treatments were stopped at three days rather than five, due to the
172 extreme gill resorption in the T3 + Bex animals. Three independent clutches of NF 54 tadpoles were used
173 with 4-5 tadpoles per clutch.
174
175 **Immunohistochemistry of lower jaws for proliferation:**
176 The lower jaws from tadpoles fixed as for morphology were removed as follows: a straight cut
177 was made just posterior to the olfactory epithelium and anterior to the eyes. the upper and lower jaw were
178 separated, and two diagonal cuts were made on the outer rim of the jaw to separate the cartilage from the
179 excess tissue. LJs were treated as described for immunohistochemical analysis of phospho-Histone H3
180 reactivity (EMD Millepore, 06-570, 1/300 dilution) (48,58). Anti-phospho-Histone H3 (Ser10) was from
181 EMD Millepore (06-570, 1/300 dilution), and goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor
182 488 was from Molecular Probes (A11008, 1/400 dilution). Positive cells were counted from blinded
183 images using the Cell Counter tool of Fiji.
184
185 **Gene Expression:**
186 Tadpoles were treated with ligands for 48 hours as for morphology and as described (47,50),
187 using a 2-way ANOVA design: vehicle (DMSO), T3, RXR ligand, and T3 + RXR ligand. Lower jaws
188 were isolated from unfixed tadpoles as for immunohistochemistry. Pools of 15 LJs from a single clutch
189 were used for total RNA extraction. LJ tissue was disrupted and homogenized by bead beating with two
190 0.125-inch stainless steel beads for 1 minute in a Mini-Beadbeater-16 (Biospec Products, Bartlesville,
191 OK). Total RNA was extracted using the RNeasy Plus Mini Kit per the manufacturer's instructions
192 (Qiagen, Germantown, MD). Total RNA was quantified using a NanoDrop (Thermo Fisher Scientific,
193 Waltham, MA). One microgram of total RNA was used to synthesize cDNA with the High-Capacity
194 Reverse Transcription Kit (Thermo Fisher Scientific), and 0.5 µl of cDNA from a 20-µl reaction was used
195 in a 10-µl reaction using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) in a Roche
196 LightCycler 480. The *X. laevis rpl8* gene was used as a normalizer. Statistics were performed using 2-way

ANOVA analysis with a Sidak's multiple comparison test (MCT) in GraphPad Prism 9. Sequences for the primers used for quantitative PCR are given S1 Table.

Transgenic tadpole luciferase reporter assay:

NF 54 tadpoles, sorted at 1wk-PF for GFP+ expression in the eye lens, were staged by assessing morphology of the hind limb, according to the normal scale by Nieuwkoop and Faber (59), and then treated through their rearing water for two days as previously described (50). Treatment concentrations were 10 nM T3 and 2 nM TBT. No mortality arose from the treatments over the treatment period. After treatment, tadpoles were anesthetized in 0.1% MS-222 (Western Medical) buffered with 0.1% sodium bicarbonate. The LJs were excised and minced on ice prior to freezing and then processed and assayed as described. Each animal was treated as an individual for statistical purposes (n = 9 per treatment) from two independent clutches (4 animals in one clutch and 5 in the other). Two-way ANOVA using clutch and treatment as covariates with Tukeys MCT to compare treatments was used from GraphPad Prism 9.

Results:

RXR agonists potentiate T3-induced morphological changes to the lower jaw, and an RXR antagonist abrogates T3 effects

Using our precocious metamorphosis assay system, we treated *X. laevis* 1wk-PF tadpoles (NF 48) for five days by exposure through their rearing water with vehicle or 10 nM T3 in the presence or absence of RXR ligands. This treatment period did not result in animal mortality under any of the treatment conditions. Previously, we found that 30 nM Bex and 1 nM TBT produced maximal, non-toxic responses, and so we used them here (47,50). The dorsal head photos in Figure 1 show representative animals from each treatment regimen. Vehicle treatment resulted in normal tadpole morphology (Figure 1a), and treatment with the RXR ligands in the absence of T3 (Figure 1b-1d) did not result in morphological changes. Treatment with 10 nM T3 (Figure 1e) resulted in visible gill resorption and decreased the angle of Meckels cartilage. Co-treatment with either RXR agonist, Bex or TBT, potentiated the T3-inductions of gill resorption and the angle of Meckels cartilage (Figure 1f-1g). However, co-treatment with the RXR antagonist UVI 3003, abrogated the effect of T3 on both morphologic phenotypes.

Figure 1: RXR agonists potentiate T3-induced changes to lower jaw morphology, while an RXR antagonist abrogates T3 action. a-h: Representative dorsal head photos of tadpoles treated for five days starting at 1wk-PF (30 nM Bex, 1 nM TBT, 600 nM UVI). i-k: Quantification of changes to the jaw angle. Boxes represent 25^{th}-75^{th} percentiles with the line at the median (n = 10-15 from 2-3 clutches), and whiskers represent the min and max values. Statistics show results from Sidak's multiple comparison test in conjunction with 2-way ANOVA (****, $p < 0.0001$). l: Effect of Bex and TBT on T3-induced jaw angle changes as a function of time. Data points represent means from 20 animals from two different clutches; error bars delineate the 95% confidence intervals, indicating statistical significance. m: Treatment with 30 nM Bex augments jaw angle narrowing as a function of T3 dose. Statistics are the same as in the time course, although the clutches were different.

In order to quantify the effects of T3 and the RXR ligands on Meckels cartilage, we measured the angle of the LJ (Figure 1i-k) from independent clutches of tadpoles, using ten animals per clutch. The inset photos (Figure 1a, 1e, 1f) show the change in angle that was measured. Protrusion of the Meckels cartilage caused a decrease in the LJ angle. Figure 1i shows that in the presence of T3, 30 nM Bex significantly potentiated the decrease in the LJ angle (compare red boxes). 2-way ANOVA analysis indicated significance for the interaction between T3 and Bex ($p < 0.0001$. As with our study on the effects of RXR agonists on T3-induced tail resorption, 1 nM TBT behaved almost identically to 30 nM Bex; the interaction between T3 and TBT was significant ($p < 0.0001$). In contrast, co-treatment of T3 and the RXR antagonist UVI prevented T3 action, and the LJ angle was not significantly changed from vehicle-treated tadpoles (Figure 1k); however, due to the strong abrogation of the T3-induction by UVI, the interaction between T3 and T3+UVI was still significant by 2-way ANOVA ($p < 0.0001$). Figure 1l shows the LJ angle measurement as a function of treatment time. Again, co-treatment of either Bex or TBT with T3 caused an identical response that showed an acceleration of the Meckels cartilage protrusion. Tadpoles treated for four days with T3 plus RXR agonist had the same decrease in LJ angle as tadpoles treated for five days with T3-alone. Over a T3-dose curve, the T3-induced decrease in LJ angle was significant starting at 5 nM T3 (error bars represent the 95% confidence interval), and all doses of T3 in the presence of Bex showed a significantly reduced LJ angle compared to T3-alone, such that 5 nM T3 plus Bex/TBT produced the same LJ angle as 15 nM T3 (Figure 1m), which is the dose that produces the maximal change in LJ angle.

RXR agonists potentiated T3-induced cellular proliferation in Meckels cartilage, but the RXR antagonist had no effect

In young tadpoles, exogenous T3 administration triggers proliferation in several tissues, including the LJ (58). We excised LJs after four days of treatment for whole mount immunohistochemistry (IHC) of the mitotic marker phosopho-Ser10 Histone 3 (pH3) to assess the effects of T3 and RXR ligands on

cellular proliferation in Meckels cartilage. 1wk-PF tadpoles were treated for four days instead of five to facilitate LJ removal; T3-induced changes to the gills and brain make removing the LJ more difficult after five days of treatment. Proliferative cells were counted from blinded images over the area of Meckels cartilage (Figure 2a). Figure 2b-f show representative photos of different ligand treatment combinations from which proliferative cells were counted. For quantification, each combination of T3 and RXR ligand were assayed with two independent clutches, and for each clutch, RXR agonist potentiation was significant. Figure 2g-i shows the two clutches combined for each group. Vehicle-treated LJs had few proliferative cells (Figure 2g-i). In contrast, treatment with 10 nM T3 increased the number of mitotic cells at least 15-fold for each treatment group. Co-treatment of either 30 nM Bex (Figure 2g) or 1 nM TBT (Figure 2h) RXR agonists with the T3 resulted in a significant increase in the number of proliferating cells in the Meckels cartilage. Since the RXR agonists induced a significant increase in proliferative cells, we expected that co-treatment of the RXR antagonist UVI with T3 would result in a decrease in proliferative cells. However, as Figure 2i shows, that is not the case; UVI did not inhibit cellular proliferation in Meckels cartilage. Therefore, the RXR agonists potentiated both the decrease in LJ angle and the induction of cell division; however, UVI prevented T3 action morphologically (Figure 1h) but had no effect on T3-induction of proliferation (Figure 2i). Aurora kinase B (*aurkb*) is the kinase that performs the phosphorylation of Ser10 of H3. T3 induced *aurkb* mRNA expression (figure 2j-l); however, neither Bex (Figure 2j) nor TBT (Figure 2k) significantly increased that induction, suggesting that increased *aurkb* expression alone was not the mechanism through which the RXR agonists potentiated cell proliferation in Meckels cartilage. UVI inhibition of *aurkb* was not significantly different from T3-alone ($p = 0.081$) (Figure 2l).

Figure 2: RXR agonists potentiate T3 action on cellular proliferation in the LJ of 1wk-PF tadpoles. a: Region of Meckels cartilage used for quantitation of proliferation. b-f: Representative photos of the effects of different treatments on proliferation using phopho-Ser10-H3 reactivity. g-i: Quantification of proliferation in the presence and absence of T3 and RXR ligands. Boxes and statistics are as in Figure 1

(n = 20-30 jaws from 2-3 clutches). j-l: RXR ligands do not significantly affect the T3-induced expression of aurora kinase B mRNA (*aurkb*). Bars represent the mean of 3-6 independent clutches, and statistics show results from Sidak's multiple comparison test in conjunction with 2-way ANOVA (****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.05$).

RXR agonist potentiation of gene expression is gene specific

Our previous work examining the role of RXR ligands to perturb T3-mediated gene expression in the tails of 1-wk-PF tadpoles after a 48-hour induction, showed that the *bona fide* TR target gene for TRβ, *thrb*, was modestly, but significantly, potentiated by the RXR agonists and inhibited by the antagonist when assayed at the transcriptomic level using Tag-Seq. However, over a time course assayed by RT-qPCR, the same two-day time point showed no significant potentiation and inhibition by the agonists and antagonist, respectively (50). Using RT-qPCR to assess *thrb* expression in the LJ after two days of treatment, we found significant activation by T3 (white bars in Figure 3a), but neither Bex nor TBT potentiated that induction (slashed bars in Figure 3a, Bex, TBT). UVI also did not inhibit the T3 induction (slashed bar in Figure 3a, UVI). TH-bZIP is a transcription factor that is one of the most strongly TH-induced genes during metamorphosis. It is encoded by the *thibz* gene, and it is another TR direct target gene, having at least two TREs in the promoter region (60). In the LJ, T3 strongly induced *thibz* expression (Figure 3b, white bars), but the RXR agonists did not potentiate the signal (Figure 3b, slashed bars, Bex, TBT). However, UVI did significantly reduce the T3 induction of *thibz* (Figure 3b, slashed bar, UVI). In the tail, we found the same outcome: the RXR agonists did not affect *thibz* expression, but the RXR antagonist significantly did (47,50). These results strongly suggest that the RXR agonists and antagonist are not always operating reciprocally.

Figure 3: RXR ligands have gene-specific effects on T3-induced differential gene expression. Left column: The effect of RXR agonist Bex on T3-induced gene expression. Middle column: The effect of environmental RXR agonist TBT on T3-induced genes. Right column: The effect of RXR antagonist UVI

316 on T3-induced genes. Striped bars indicate the presence of the RXR ligand, and white bars show
317 induction in the absence of the RXR ligand. Statistics show results from Sidak's multiple comparison test
318 in conjunction with 2-way ANOVA (****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).
319
320 During metamorphosis, matrix metalloprotease activity is essential for both tissue resorption and
321 tissue remodeling. We and others have shown the importance of stromelysin-3 (*mmp11*) and collagenase-
322 3 (*mmp13l*) expression (61–63). We found in the LJ that *mmp11* was strongly activated by T3 (white bars
323 in Figure 3c). Co-treatment with 30 nM Bex increased *mmp11* expression, although this result did not
324 reach statistical significance ($p = 0.068$), using the maximal number of biological replicates ($n = 6$)
325 recommended for pooled, outbred animal tissues. However, co-treatment of T3 with 1 nM TBT did result
326 in significantly potentiation of *mmp11* expression ($n = 3$, $p = 0.0004$). Furthermore, UVI inhibited the T3
327 induction of *mmp11* significantly ($n = 4$, $p = 0.001$). In the experiments using Bex, even though T3 on its
328 own activated the *mmp13l* gene 20.8-fold (S.E.M. = 4.95) (white bars in Figure 3d), this activation did not
329 reach statistical significance ($p = 0.0589$), like it does in the tail. However, co-treatment with Bex
330 increased *mmp13l* activation to significance ($p < 0.0001$) compared to both vehicle and T3-only
331 treatments (slashed bar in Figure 3d, Bex). This situation held true for co-treating T3 with TBT (slashed
332 bar in Figure 3d, TBT): T3-alone activation of *mmp13l* was not significant ($p = 0.65$), while T3 + TBT
333 treatment was significantly potentiated ($p < 0.0001$) compared to both vehicle and T3-alone (slashed bar
334 in Figure 3d, TBT). In contrast, in the experiments using UVI, the 7.3-fold activation by T3-alone did
335 reach statistical significance ($p = 0.0008$, $n = 4$), but UVI did not significantly inhibit T3 induction of the
336 gene ($p = 0.14$).
337 Runx2 is a transcription factor that is required for the transition from proliferating chondrocytes
338 to hypertrophic chondrocytes in the maturation of cartilage for the development of a bony skeleton (64–
339 66). In non-amniote animals like fish and amphibia, it is required earlier for rostral cartilage formation
340 (67,68). Due to the extensive changes to jaw cartilage during metamorphosis, we investigated whether T3
341 regulated its expression. In the LJ, T3 induced expression of *runx2* approximately 7-fold (white bars in

Figure 3e), and this induction was significantly potentiated through co-treatment of either Bex or TBT with the T3 (slashed bars in Figure 3e, Bex, TBT). In addition, UVI co-treatment significantly inhibited *runx2* induction by T3 (slashed bar in Figure 3e, UVI). T3 did not regulate the expression of *runx3*, nor did we see activation of certain *runx2* downstream targets like *col10a1* (collagen10α1) (data not shown).

RXR agonists potentiate T3-action in TH-competent (NF 54) tadpoles

While 1wk-PF tadpoles are considered only partially competent to respond to THs, tadpoles at NF 54 (approximately 26 days PF) are considered fully competent to respond to THs and to be entering metamorphosis (21,49,61). We raised tadpoles to NF 54, using hind limb development to determine the developmental stage (59), and then treated them with 10 nM T3 in the presence and absence of 30 nM Bex to investigate whether the RXR agonist could still potentiate the action of T3 in a fully competent tadpole. Tadpoles were treated for three days with compounds (a longer treatment time was not possible due to the extreme gill resorption in T3 plus Bex animals), and then we measured the LJ angle. Figure 4a (white boxes) shows that T3-alone caused a small but significant decrease in the lower jaw angle. As in NF 48 tadpoles, Bex-only treatment had no effect on the lower jaw morphology—tadpoles were indistinguishable from vehicle-treated. Bex co-treatment with T3 significantly potentiated the decrease in the LJ angle at this later stage of growth (Figure 4a), suggesting that the ability to increase the competence for T3 in the lower jaw was still possible, even for these presumed fully competent animals.

Figure 4: RXR agonists potentiate T3 action in the LJ in pro-metamorphic NF 54 tadpoles. a. Bex potentiates the T3-induced decrease in the LJ angle in NF 54 tadpoles treated for three days. Boxes and statistics are as in Figure 1 (n = 14 jaws from 3 clutches). Statistics show results from Sidak's multiple comparison test in conjunction with 2-way ANOVA (****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.05$). b. TBT potentiates T3-inducible, integrated luciferase reporter expression in the LJ of NF 54 tadpoles.

Previously we developed a transgenic line of *X. laevis* frogs that express firefly luciferase (Luc) under the regulation of the *X. laevis thibz* TH response elements (TREs) (47,48). In NF 48 tadpoles, assaying the entire head for Luc activation is required in order to generate a signal robust enough for statistics. At NF 54 we are able to analyze individual tissues, so we treated NF 54 tadpoles for 2 days with 10 nM T3 in the presence and absence of 2 nM TBT, and then we excised the lower jaws as we did for gene expression analysis. Luc activity was determined in the lower jaw samples and was normalized to the protein concentration of each sample. We assayed two clutches independently using two different TRE-Luc-bearing F2 male frogs to generate embryos with two different wild-type female frogs. TRE-Luc F2 males, even though they arise from the same founder female, display different levels of Luc activation by T3 that are nonetheless consistent within a clutch. Figure 4B shows the results of both clutches individually, showing the different levels of T3 activation between the two clutches. For clutch 2, a TBT-only treatment was also included and showed no activation. Using a 2-way ANOVA analysis of the combined data from both clutches where treatment and clutch were covariates, clutch was a significant source of variance ($p = 0.0005$), as was treatment ($p < 0.0001$). Using a Tukey multiple comparisons test post hoc on the combined clutch data, TBT significantly potentiated the T3 activation of the Luc reporter ($p = 0.0092$). This result indicates that the RXR agonists at this high-TH-competence stage could further increase the competence of LJ tissue for T3 at the beginning of natural metamorphosis.

Discussion

In this report we have expanded upon our earlier findings concerning the ability of RXR agonists to function as a competence factor for TH signaling during vertebrate development (47,48,50). The poor biological outcomes that arise from insufficient or inappropriate TH during development have demonstrated the need for assessing the ability of man-made chemicals present in the environment to disrupt those signaling pathways.

In order to look at TH disruption in vivo and during development, we have used amphibian metamorphosis of the African clawed frog, *Xenopus laevis*. Metamorphosis performs two reciprocal functions: 1) development of adult tissues and organs required for life as a frog, and 2) removal of larval tissues no longer needed by the adult frog. Limb formation and growth and lung development are examples of development of new tissues and organs, and jaw development is an example of remodeling that must occur for the herbivorous tadpole to become a carnivorous frog. The other side of the metamorphic coin involves the resorption of larval tissues that are no longer required in the frog, such as gills and the tail. Naturally, removal of larval tissues must occur after the adult tissues have developed and become functional. For example, tail resorption is the last step in metamorphosis because it must occur after limb development is complete and the limbs are functional for locomotion. Under natural development, it takes approximately two months to go from a fertilized egg through a larval tadpole to a juvenile frog, with the metamorphic transition from tadpole to frog taking approximately 4.5 weeks under ideal conditions (13,18,21,59).

Our studies here employed a precocious metamorphosis assay, to determine whether a disruptor of TH signaling, which we have previously described disrupting larval tissue resorption phenotypes (47,50), can also disrupt a larval-to-adult remodeling function, namely, cartilage development in the LJ. By using 1wk-PF tadpoles, we were able to control the dose of TH, as tadpoles at this age do not yet synthesize THs. In this scenario, T3 and the potential disrupting chemicals taken up by the tadpole through administration in the rearing water. Although the LJ of the 1wk-PF tadpole is not able to support

normal metamorphic changes to the LJ, molecularly the LJ can respond to T3 administration with reproducible morphological and molecular readouts.

Previously, we found that both the pharmaceutical RXR agonist Bex and the environmental RXR agonist TBT disrupted TH signaling in 1wk-PF tadpoles by significantly potentiating the ability of T3 to drive gill and tail resorption. Furthermore, the RXR antagonist UVI abrogated T3 action. Bex and TBT functioned identically in a global transcriptomic analysis of T3 signaling in the tail (50), indicating that TBT was functioning as a bona fide RXR agonist (43,44,46,69–72). Here, we show that the RXR agonists potentiate T3 action in the LJ by accelerating the rate of change and by increasing the potency of each T3 dose. As in the tail, TBT and Bex behaved nearly identically in the LJ independent of the experimental readout. In addition, the RXR antagonist abrogated the morphological changes induced by T3. We also measured the ability of the agonists and antagonist to disrupt T3-induced cellular proliferation. TBT and Bex both significantly potentiated proliferation, but UVI did not inhibit it. These findings suggest that the mechanisms of RXR agonist potentiation and of RXR antagonist inhibition are not strictly reciprocal. Furthermore, in contrast to their effects on proliferation, the opposite was seen in their effects on the T3-induction of the *thibz* gene. There, the RXR agonists had no effect, and the RXR antagonist significantly inhibited *thibz* activation. This was also seen in tail expression of *thibz* (50). How the agonists and antagonist are working at the molecular level is beyond the scope of these studies, but more than one mechanism is in play. Interestingly, RXR agonist s and the antagonist do not always behave in a reciprocal manner at all molecular or cellular targets when examined in detail.

UVI prevented morphological changes to the LJ in the presence of T3 but did not inhibit cellular proliferation, which suggests that cellular proliferation was not the main driver behind the morphological narrowing of the LJ. A better fit to the morphology patterns observed with RXR agonists and antagonist modulation of the LJ T3 response is the expression patterns of the matrix metalloproteases we tested. Both *mmp11* and *mmp13l* expression levels were potentiated by RXR agonists and inhibited by UVI. This was also true for the transcription factor *runx2*, which in mice is required for formation of ossified bones (73). In *Xenopus* and zebrafish, *runx2* is required earlier for cranial cartilage formation (67,68), but in our

hands, it was significantly activated by T3 exposure, and that activation was potentiated by the RXR agonists and inhibited by UVI. We believe this is the first example of T3 activating *runx2* expression. In human thyroid cancer and breast cancer cells, TRβ suppressed the expression of *runx2* in the presence of TH, acting as a tumor suppressor (74,75).

An advantage of using 1wk-PF tadpoles for characterizing disruptors of TH signaling is the size uniformity of the tadpoles. We normally don't have to normalize to the vehicle-treated control in each clutch, as we didn't in Figure 1. However, as the tadpoles age, this size uniformity disappears, making morphological measurements more intrusive, as the animals must be housed separately and anesthetized and photographed before treatment for individual comparisons to after treatment changes. An advantage of assaying the LJ angle, is that it does not scale with tadpole head size; therefore, tadpoles can be group housed and measured only after fixation at the end of treatment. This provides a facile assay for TH disruption over developmental time, which in the case of RXR ligands, as they affect TH competence, could change as the animal develops and intrinsically increases in TH competence.

That said, we also chose NF 54 to assess whether the RXR agonists could still potentiate T3 action in the LJ because that is when plasma T3 is first detectable, and therefore, NF 54 is often considered the dividing line between premetamorphic and metamorphic tadpoles (21). However, NF 54 is nearly three weeks before metamorphic morphological changes in the jaw become apparent at NF 59 (51), and it is approximately two weeks before exogenous T3 leads to normal metamorphic development in the LJ. Therefore, TH competence in the LJ may still not be complete at NF 54 so that the cartilages can continue to develop in their normal T3-independent fashion until they are in the form that can remodel appropriately to an adult jaw. Thus, as prometamorphosis proceeds, the animal may be vulnerable to inappropriate RXR ligand activity from the environment. Ordinarily, endogenous retinoids can be controlled by the P450 retinoid-degrading enzymes, (76,77), yet organotins, or other as yet unknown chemicals in the environment that activate RXR, evade this buffer, and, therefore, still pose a unique and challenging problem for the exquisitely timed process of metamorphosis.

Acknowledgements:

The authors would like to acknowledge Michael L. Goodson for blinding the images for counting proliferative cells.

References

1. Leitch VD, Bassett JHD, Williams GR. Role of thyroid hormones in craniofacial development. Nat Rev Endocrinol [Internet]. 2020;16(3):147–64. Available from: http://dx.doi.org/10.1038/s41574-019-0304-5

2. Duncan Bassett JH, Williams GR. Role of thyroid hormones in skeletal development and bone maintenance. Endocr Rev. 2016;37(2):135–87.

3. Gouveia CHA, Miranda-Rodrigues M, Martins GM, Neofiti-Papi B. Thyroid Hormone and Skeletal Development. Vitam Horm [Internet]. 1st ed. 2018;106:383–472. Available from: http://dx.doi.org/10.1016/bs.vh.2017.06.002

4. Lindsey RC, Aghajanian P, Mohan S. Thyroid Hormone Signaling in the Development of the Endochondral Skeleton [Internet]. 1st ed. Vol. 106, Vitamins and Hormones. Elsevier Inc.; 2018. 351–381 p. Available from: http://dx.doi.org/10.1016/bs.vh.2017.06.003

5. Bianco AC, Dumitrescu A, Gereben B, Ribeiro MO, Fonseca TL, Fernandes GW, et al. Paradigms of Dynamic Control of Thyroid Hormone Signaling. Endocr Rev [Internet]. 2019;40(4):1000–47. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31033998

6. Fan X, Wu L. The impact of thyroid abnormalities during pregnancy on subsequent neuropsychological development of the offspring: a meta-analysis. J Matern Neonatal Med. 2016;29(24):3971–6.

7. Forhead AJ, Fowden AL. Thyroid hormones in fetal growth and prepartum maturation. J Endocrinol [Internet]. 2014 Jun;221(3):R87–103. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24648121

8. Patel J, Landers K, Li H, Mortimer RH, Richard K. Thyroid hormones and fetal neurological development. J Endocrinol. 2011;209(1):1–8.

9. Labadzhyan A, Brent GA, Hershman JM, Leung AM. Thyrotoxicosis of Pregnancy. J Clin Transl Endocrinol [Internet]. 2014 Dec 1;1(4):140–4. Available from: http://dx.doi.org/10.1016/j.jcte.2014.07.008

10. Nunez J, Celi FS, Ng L, Forrest D. Multigenic control of thyroid hormone functions in the nervous system. Mol Cell Endocrinol [Internet]. 2008 Jun 11;287(1–2):1–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18448240

11. Buchholz DR. More similar than you think: Frog metamorphosis as a model of human perinatal endocrinology. Dev Biol [Internet]. 2015;408(2):188–95. Available from: http://dx.doi.org/10.1016/j.ydbio.2015.02.018

12. Grimaldi A, Buisine N, Miller T, Shi Y-B, Sachs LM. Mechanisms of thyroid hormone receptor action during development: lessons from amphibian studies. Biochim Biophys Acta [Internet]. 2013 Jul;1830(7):3882–92. Available from: http://dx.doi.org/10.1016/j.bbagen.2012.04.020

13. Furlow JD, Neff ES. A developmental switch induced by thyroid hormone: Xenopus laevis metamorphosis. Trends Endocrinol Metab. 2006;17(2):38–45.

14. Sachs LM, Buchholz DR. Frogs model man: In vivo thyroid hormone signaling during development. Genesis [Internet]. 2017 Jan;55(1–2):1–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28109053

15. Das B, Matsuda H, Fujimoto K, Sun G, Matsuura K, Shi Y-B. Molecular and genetic studies suggest that thyroid hormone receptor is both necessary and sufficient to mediate the developmental effects of thyroid hormone. Gen Comp Endocrinol [Internet]. 2010 Sep 1;168(2):174–80. Available from: http://dx.doi.org/10.1016/j.ygcen.2010.01.019

16. Buchholz DR, Tomita A, Fu L, Paul BD, Shi Y. Transgenic Analysis Reveals that Thyroid Hormone Receptor Is Sufficient To Mediate the Thyroid Hormone Signal in Frog Metamorphosis. Mol Cell Biol. 2004;24(20):9026–37.

17. Das B, Heimeier RA, Buchholz DR, Shi Y. Identification of direct thyroid hormone response genes reveals the earliest gene regulation programs during frog metamorphosis. J Biol Chem. 2009;284(49):34167–78.

18. Brown DD, Cai L. Amphibian metamorphosis. Dev Biol [Internet]. 2007 Jun 1;306(1):20–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17449026

19. Mendoza A, Hollenberg AN. New insights into thyroid hormone action. Pharmacol Ther [Internet]. 2017;173:135–45. Available from: http://dx.doi.org/10.1016/j.pharmthera.2017.02.012

20. Brent GA. Mechanisms of thyroid hormone action. J Clin Invest [Internet]. 2012 Sep;122(9):3035–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22945636

21. Shi Y-B. Life Without Thyroid Hormone Receptor. Endocrinology [Internet]. 2021 Apr 1;162(4):1–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33558878

22. Kanamori A, Brown DD. The regulation of thyroid hormone receptor beta genes by thyroid hormone in Xenopus laevis. J Biol Chem [Internet]. 1992;267(2):739–45. Available from: http://www.jbc.org/content/267/2/739.abstract

23. Wong J, Liang VCT, Sachs LM, Shi YB. Transcription from the thyroid hormone-dependent promoter of the Xenopus laevis thyroid hormone receptor βA gene requires a novel upstream element and the initiator, but not a TATA box. J Biol Chem [Internet]. 1998;273(23):14186–93. Available from: http://dx.doi.org/10.1074/jbc.273.23.14186

24. Galton VA. The ups and downs of the thyroxine pro-hormone hypothesis. Mol Cell Endocrinol [Internet]. 2017 Dec 15;458:105–11. Available from: http://dx.doi.org/10.1016/j.mce.2017.01.029

25. Oppenheimer JH, Schwartz HL, Surks MI. Propylthiouracil inhibits the conversion of L-thyroxine to L-triiodothyronine. An explanation of the antithyroxine effect of propylthiouracil and evidence supporting the concept that triiodothyronine is the active thyroid hormone. J Clin Invest. 1972;51(9):2493–7.

26. Samuels HH, Tsai JS. Thyroid hormone action. Demonstration of similar receptors in isolated nuclei of rat liver and cultured GH1 cells. J Clin Invest. 1974;53(2):656–9.

27. Puzianowska-Kuznicka M, Damjanovski S, Shi YB. Both thyroid hormone and 9-cis retinoic acid receptors are required to efficiently mediate the effects of thyroid hormone on embryonic development and specific gene regulation in Xenopus laevis. Mol Cell Biol [Internet]. 1997 Aug;17(8):4738–49. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9234730

28. Dawson MI, Xia Z. The retinoid X receptors and their ligands. Biochim Biophys Acta [Internet].

2012 Jan;1821(1):21–56. Available from:
http://linkinghub.elsevier.com/retrieve/pii/S1388198111001843

29. Yen PM, Sugawara A, Chin WW. Triiodothyronine (T3) differentially affects T3-receptor/retinoic acid receptor and T3-receptor/retinoid X receptor heterodimer binding to DNA. J Biol Chem [Internet]. 1992 Nov 15;267(32):23248–52. Available from:
http://www.ncbi.nlm.nih.gov/pubmed/1331079

30. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, et al. Executive Summary to EDC-2: The Endocrine Society's second Scientific Statement on endocrine-disrupting chemicals. Endocr Rev. 2015;36(6):593–602.

31. Freitas J, Cano P, Craig-Veit C, Goodson ML, David Furlow J, Murk AJ. Detection of thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay. Toxicol Vitr [Internet]. 2011;25(1):257–66. Available from: http://dx.doi.org/10.1016/j.tiv.2010.08.013

32. Freitas J, Miller N, Mengeling BJBJ, Xia M, Huang R, Houck K, et al. Identification of thyroid hormone receptor active compounds using a quantitative high-throughput screening platform. Curr Chem genomics Transl Med [Internet]. 2014;8(1):36–46. Available from:
http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3999704&tool=pmcentrez&rendertype=abstract

33. Mengeling BJ, Furlow JD. Pituitary specific retinoid-X receptor ligand interactions with thyroid hormone receptor signaling revealed by high throughput reporter and endogenous gene responses. Toxicol In Vitro [Internet]. 2015 Oct;29(7):1609–18. Available from:
http://dx.doi.org/10.1016/j.tiv.2015.06.018

34. Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, et al. Retinoic acid receptors and retinoid X receptors: Interactions with endogenous retinoic acids. Proc Natl Acad Sci U S A. 1993;90(1):30–4.

35. Evans RM, Mangelsdorf DJ. Nuclear receptors, RXR, and the big bang. Cell [Internet]. 2014;157(1):255–66. Available from: http://dx.doi.org/10.1016/j.cell.2014.03.012

573 36. Forman BM, Umesono K, Chen J, Evans RM. Unique response pathways are established by
574 allosteric interactions among nuclear hormone receptors. Cell [Internet]. 1995 May 19;81(4):541–
575 50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7758108
576 37. Castillo AI, Sánchez-martínez R, Moreno JL, Martínez-iglesias OA, Palacios D, Aranda A, et al.
577 A Permissive Retinoid X Receptor / Thyroid Hormone Receptor Heterodimer Allows Stimulation
578 of Prolactin Gene Transcription by Thyroid Hormone and 9- cis -Retinoic Acid. Mol Cell Biol
579 [Internet]. 2004;24(2):502–13. Available from:
580 http://www.ncbi.nlm.nih.gov/pubmed/14701725%0Ahttp://www.pubmedcentral.nih.gov/articleren
581 der.fcgi?artid=PMC343792%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=34379
582 2&tool=pmcentrez&rendertype=abstract
583 38. Golden WM, Weber KB, Hernandez TL, Sherman SI, Woodmansee WW, Haugen BR. Single-
584 dose rexinoid rapidly and specifically suppresses serum thyrotropin in normal subjects. J Clin
585 Endocrinol Metab. 2007;92(1):124–30.
586 39. Liu SHA, Ogilvie KM, Klausing KAY, Lawson MA, Jolley D, Li D, et al. Mechanism of Selective
587 Retinoid X Receptor Agonist- Induced Hypothyroidism in the Rat. Pharmacia. 2002;143(8):2880–
588 5.
589 40. Sherman SI, Gopal J, Haugen BR, Chiu AC, Whaley K, Nowlakha P, et al. Central
590 hypothyroidism associated with retinoid X receptor-selective ligands. N Engl J Med [Internet].
591 1999 Apr 8;340(14):1075–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10194237
592 41. Horiguchi T, Shiraishi H, Shimizu M, Morita M. Effects of triphenyltin chloride and five other
593 organotin compounds on the development of imposex in the rock shell, Thais clavigera. Environ
594 Pollut [Internet]. 1997;95(1):85–91. Available from:
595 http://www.ncbi.nlm.nih.gov/pubmed/15093477
596 42. Mensink BP, Kralt H, Vethaak AD, Ten Hallers-Tjabbes CC, Koeman JH, Van Hattum B, et al.
597 Imposex induction in laboratory reared juvenile Buccinum undatum by tributyltin (TBT). Environ
598 Toxicol Pharmacol. 2002;11(1):49–65.

599 43. Nishikawa J. Involvement of the Retinoid X Receptor in the Development of Imposex Caused by
600 Organotins in Gastropods. 2004;38(23):6271–6.
601 44. Urushitani H, Katsu Y, Kagechika H, Sousa ACA, Barroso CM, Ohta Y, et al. Characterization
602 and comparison of transcriptional activities of the retinoid X receptors by various organotin
603 compounds in three prosobranch gastropods; Thais clavigera, Nucella lapillus and Babylonia
604 japonica. Aquat Toxicol [Internet]. 2018 Jun;199(March):103–15. Available from:
605 https://doi.org/10.1016/j.aquatox.2018.03.029
606 45. Castro LFC, Lima D, Machado A, Melo C, Hiromori Y, Nishikawa J, et al. Imposex induction is
607 mediated through the Retinoid X Receptor signalling pathway in the neogastropod Nucella
608 lapillus. Aquat Toxicol [Internet]. 2007 Nov 15;85(1):57–66. Available from:
609 http://www.ncbi.nlm.nih.gov/pubmed/17875330
610 46. le Maire A, Grimaldi M, Roecklin D, Dagnino S, Vivat-Hannah V, Balaguer P, et al. Activation of
611 RXR-PPAR heterodimers by organotin environmental endocrine disruptors. EMBO Rep
612 [Internet]. 2009 Apr;10(4):367–73. Available from:
613 http://www.ncbi.nlm.nih.gov/pubmed/19270714
614 47. Mengeling BJ, Murk AJ, Furlow JD. Trialkyltin Rexinoid-X Receptor Agonists Selectively
615 Potentiate Thyroid Hormone Induced Programs of Xenopus laevis Metamorphosis. Endocrinology
616 [Internet]. 2016 Jul;157(7):2712–23. Available from:
617 http://www.ncbi.nlm.nih.gov/pubmed/27167774
618 48. Mengeling BJ, Wei Y, Dobrawa LN, Streekstra M, Louisse J, Singh V, et al. A multi-tiered, in
619 vivo, quantitative assay suite for environmental disruptors of thyroid hormone signaling. Aquat
620 Toxicol [Internet]. 2017 Sep;190:1–10. Available from:
621 http://www.ncbi.nlm.nih.gov/pubmed/28662416
622 49. Wang Z, Brown DD. Thyroid hormone-induced gene expression program for amphibian tail
623 resorption. J Biol Chem. 1993;268(22):16270–8.
624 50. Mengeling BJ, Goodson ML, Furlow JD. RXR Ligands Modulate Thyroid Hormone Signaling

Competence in Young Xenopus laevis Tadpoles. Endocrinology [Internet]. 2018 Jul 1;159(7):2576–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29762675

51. Rose CS, Cahill JW. How thyroid hormones and their inhibitors affect cartilage growth and shape in the frog Xenopus laevis. J Anat [Internet]. 2019;234(1):89–105. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/joa.12897

52. Rose CS, Murawinski D, Horne V. Deconstructing cartilage shape and size into contributions from embryogenesis, metamorphosis, and tadpole and frog growth. J Anat. 2015;226(6):575–95.

53. Thomson DAR. Meckel's cartilage in Xenopus laevis during metamorphosis: a light and electron microscope study. J Anat [Internet]. 1986 Dec;149:77–87. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3693112

54. Thomson DAR. A quantitative analysis of cellular and matrix changes in Meckel's cartilage in Xenopus laevis. J Anat. 1987;Vol. 151:249–54.

55. Nahoum V, Pérez E, Germain P, Rodríguez-Barrios F, Manzo F, Kammerer S, et al. Modulators of the structural dynamics of the retinoid X receptor to reveal receptor function. Proc Natl Acad Sci U S A [Internet]. 2007 Oct 30;104(44):17323–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17947383

56. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: An open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676–82.

57. Schindelin J, Rueden CT, Hiner MC, Eliceiri KW. The ImageJ ecosystem: An open platform for biomedical image analysis. Mol Reprod Dev. 2015;82(7–8):518–29.

58. Schreiber AM, Das B, Huang H, Marsh-armstrong N, Brown DD. Diverse developmental programs of Xenopus laevis metamorphosis are inhibited by a dominant negative thyroid hormone receptor. 2001;98(19):10739–44.

59. P.D. Nieuwkoop, J. Faber, John Gerhart MK. Normal Tables of Xenopus laevis (Daudin) A Systematical and Chronological Survey of the Development from the Fertilized Egg Till the End of Metamorphosis. Garland Science; 1994. 282 p.

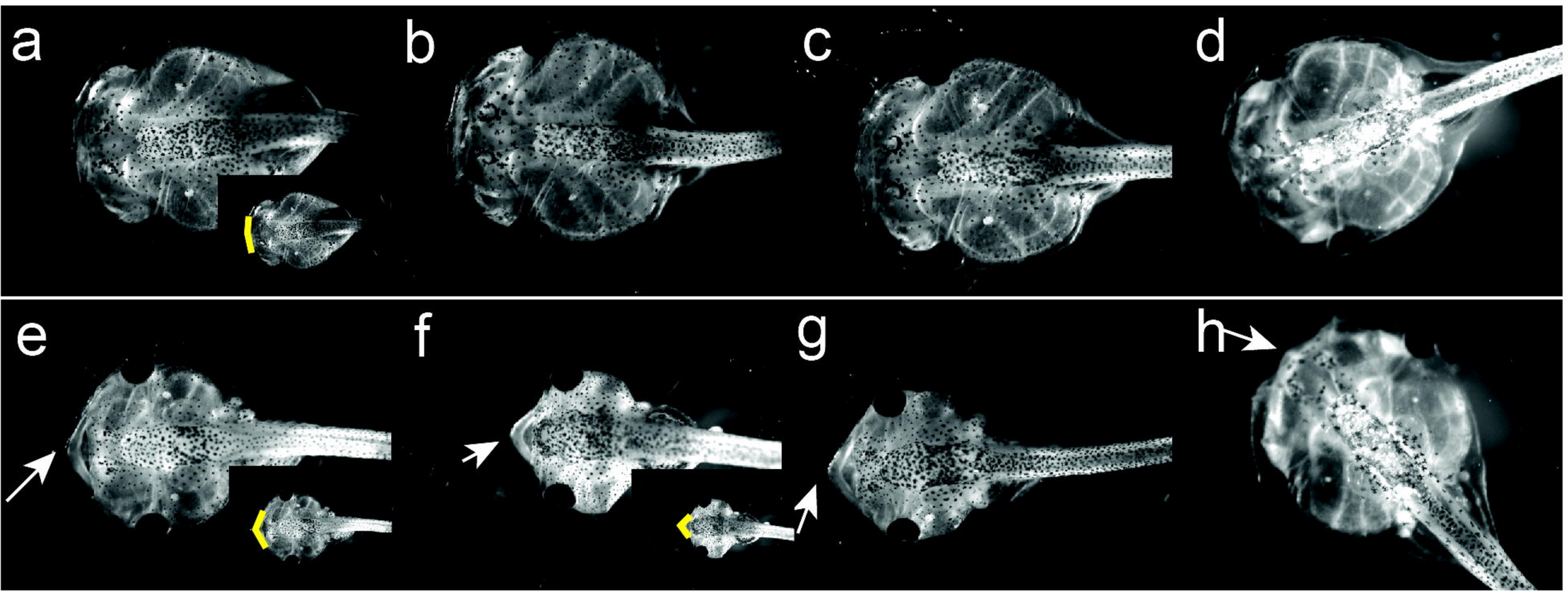
651 60. Furlow JD, Brown DD. In vitro and in vivo analysis of the regulation of a transcription factor gene
652 by thyroid hormone during Xenopus laevis metamorphosis. Mol Endocrinol. 1999;13(12):2076–
653 89.
654 61. Das B, Cai L, Carter MG, Piao YL, Sharov AA, Ko MSH, et al. Gene expression changes at
655 metamorphosis induced by thyroid hormone in Xenopus laevis tadpoles. Dev Biol.
656 2006;291(2):342–55.
657 62. Fujimoto K, Nakajima K, Yaoita Y. Expression of matrix metalloproteinase genes in regressing or
658 remodeling organs during amphibian metamorphosis. Dev Growth Differ. 2007;49(2):131–43.
659 63. Mathew S, Fu L, Fiorentino M, Matsuda H, Das B, Shi YB. Differential regulation of cell type-
660 specific apoptosis by stromelysin-3: A potential mechanism via the cleavage of the Laminin
661 receptor during tail resorption in xenopus laevis. J Biol Chem [Internet]. 2009;284(27):18545–56.
662 Available from: http://dx.doi.org/10.1074/jbc.M109.017723
663 64. Nishimura R, Hata K, Nakamura E, Murakami T, Takahata Y. Transcriptional network systems in
664 cartilage development and disease. Histochem Cell Biol [Internet]. 2018 Apr;149(4):353–63.
665 Available from: http://dx.doi.org/10.1007/s00418-017-1628-7
666 65. Komori T. Runx2, an inducer of osteoblast and chondrocyte differentiation. Histochem Cell Biol
667 [Internet]. 2018 Apr;149(4):313–23. Available from: http://dx.doi.org/10.1007/s00418-018-1640-6
668 66. Komori T. Regulation of Proliferation, Differentiation and Functions of Osteoblasts by Runx2. Int
669 J Mol Sci [Internet]. 2019 Apr 4;20(7):1694. Available from: https://www.mdpi.com/1422-
670 0067/20/7/1694
671 67. Flores MV, Lam EYN, Crosier P, Crosier K. A hierarchy of Runx transcription factors modulate
672 the onset of chondrogenesis in craniofacial endochondral bones in zebrafish. Dev Dyn.
673 2006;235(11):3166–76.
674 68. Kerney R, Gross JB, Hanken J. Runx2 is essential for larval hyobranchial cartilage formation in
675 Xenopus laevis. Dev Dyn. 2007;236(6):1650–62.
676 69. Nakanishi T, Nishikawa J, Hiromori Y, Yokoyama H, Koyanagi M, Takasuga S, et al. Trialkyltin

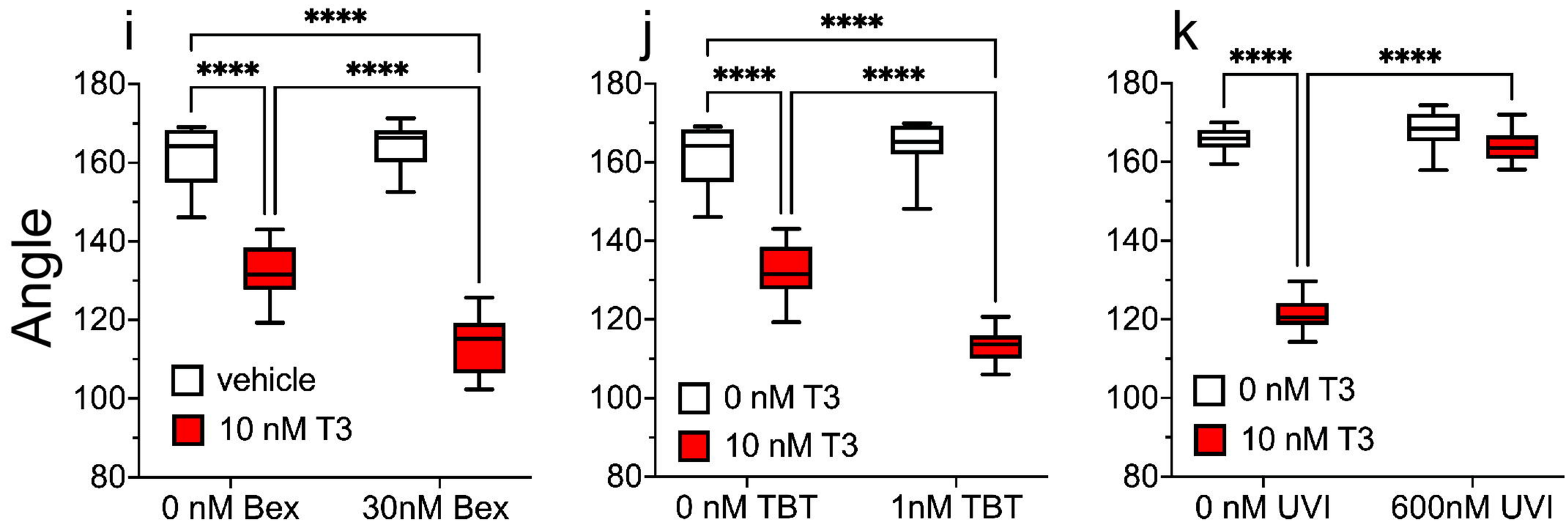
677 compounds bind retinoid X receptor to alter human placental endocrine functions. Mol Endocrinol
678 [Internet]. 2005 Oct;19(10):2502–16. Available from:
679 http://www.ncbi.nlm.nih.gov/pubmed/15941851
680 70. Shoucri BM, Hung VT, Chamorro-García R, Shioda T, Blumberg B. Retinoid X Receptor
681 Activation During Adipogenesis of Female Mesenchymal Stem Cells Programs a Dysfunctional
682 Adipocyte. Endocrinology [Internet]. 2018;159(8):2863–83. Available from:
683 http://www.ncbi.nlm.nih.gov/pubmed/29860300
684 71. Shoucri BM, Martinez ES, Abreo TJ, Hung VT, Moosova Z, Shioda T, et al. Retinoid x receptor
685 activation alters the chromatin landscape to commit mesenchymal stem cells to the adipose
686 lineage. Endocrinology. 2017;158(10):3109–25.
687 72. Hiromori Y, Aoki A, Nishikawa J, Nagase H, Nakanishi T. Transactivation of the human retinoid
688 X receptor by organotins: use of site-directed mutagenesis to identify critical amino acid residues
689 for organotin-induced transactivation. Metallomics [Internet]. 2015 Jul;7(7):1180–8. Available
690 from: http://xlink.rsc.org/?DOI=C5MT00086F
691 73. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of
692 Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts.
693 Cell [Internet]. 1997 May 30;89(5):755–64. Available from: http://dx.doi.org/10.1016/S0092-
694 8674(00)80258-5
695 74. Bolf EL, Gillis NE, Barnum MS, Beaudet CM, Yu GY, Tomczak JA, et al. The Thyroid Hormone
696 Receptor-RUNX2 Axis: A Novel Tumor Suppressive Pathway in Breast Cancer. Horm Cancer
697 [Internet]. 2020;11(1):34–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31865591
698 75. Carr FE, Tai PWL, Barnum MS, Gillis NE, Evans KG, Taber TH, et al. Thyroid Hormone
699 Receptor-β (TRβ) Mediates Runt-Related Transcription Factor 2 (Runx2) Expression in Thyroid
700 Cancer Cells: A Novel Signaling Pathway in Thyroid Cancer. Endocrinology [Internet].
701 2016;157(8):3278–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27253998
702 76. Marill J, Capron CC, Idres N, Chabot GG. Human cytochrome P450s involved in the metabolism

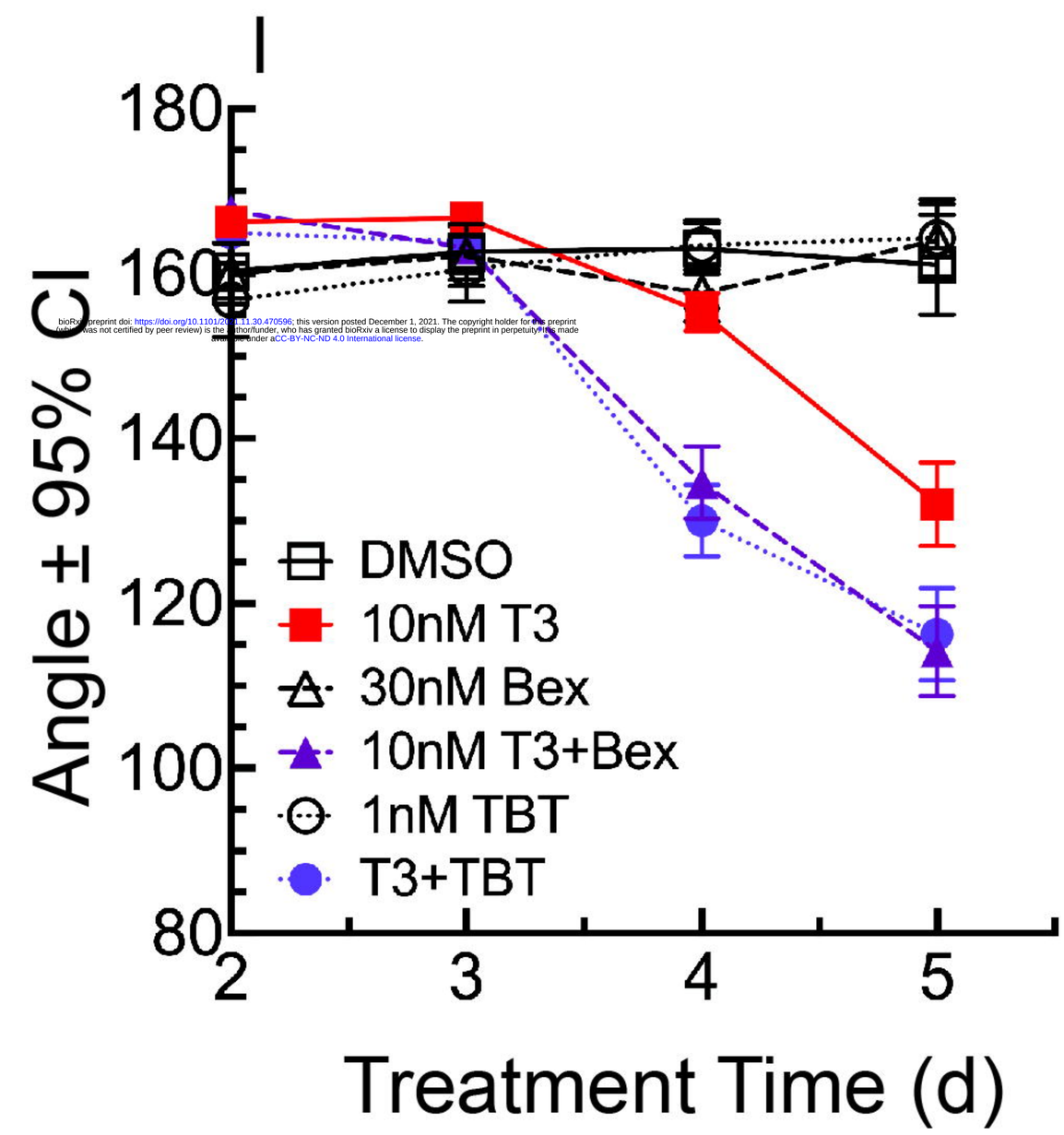
of 9-cis- and 13-cis-retinoic acids. Biochem Pharmacol [Internet]. 2002 Mar 1;63(5):933–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11911845

77. Topletz AR, Tripathy S, Foti RS, Shimshoni JA, Nelson WL, Isoherranen N. Induction of CYP26A1 by metabolites of retinoic acid: evidence that CYP26A1 is an important enzyme in the elimination of active retinoids. Mol Pharmacol [Internet]. 2015;87(3):430–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25492813

Figure 1







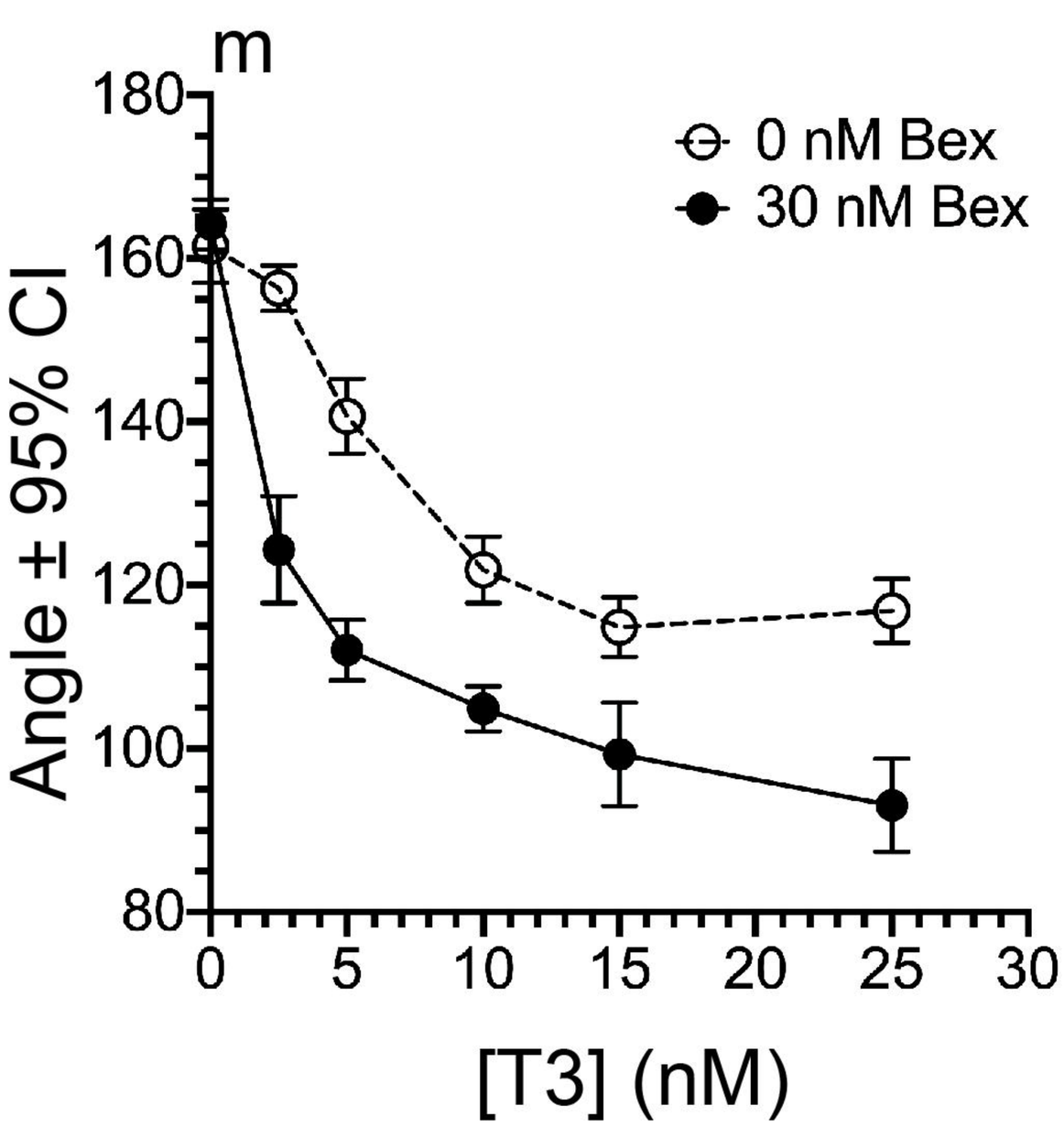
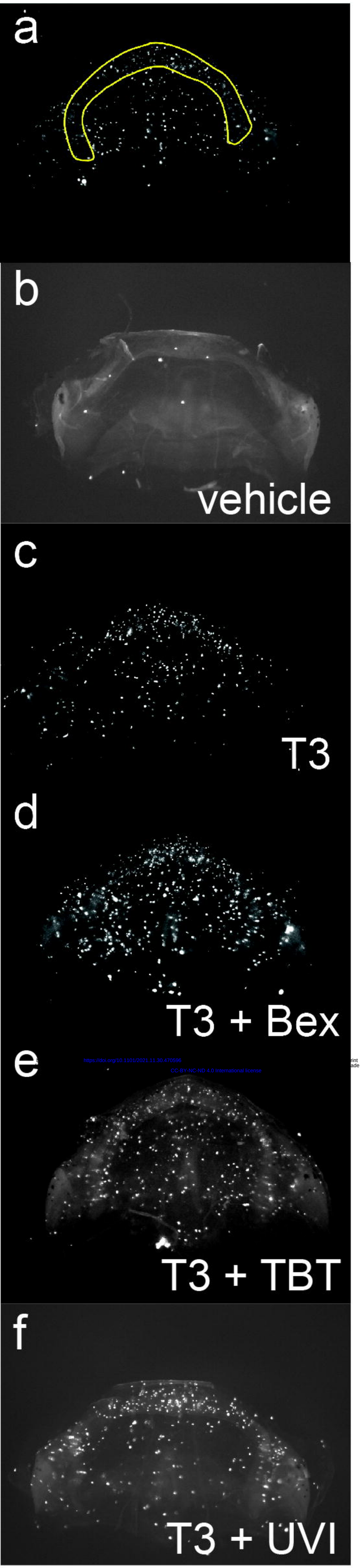
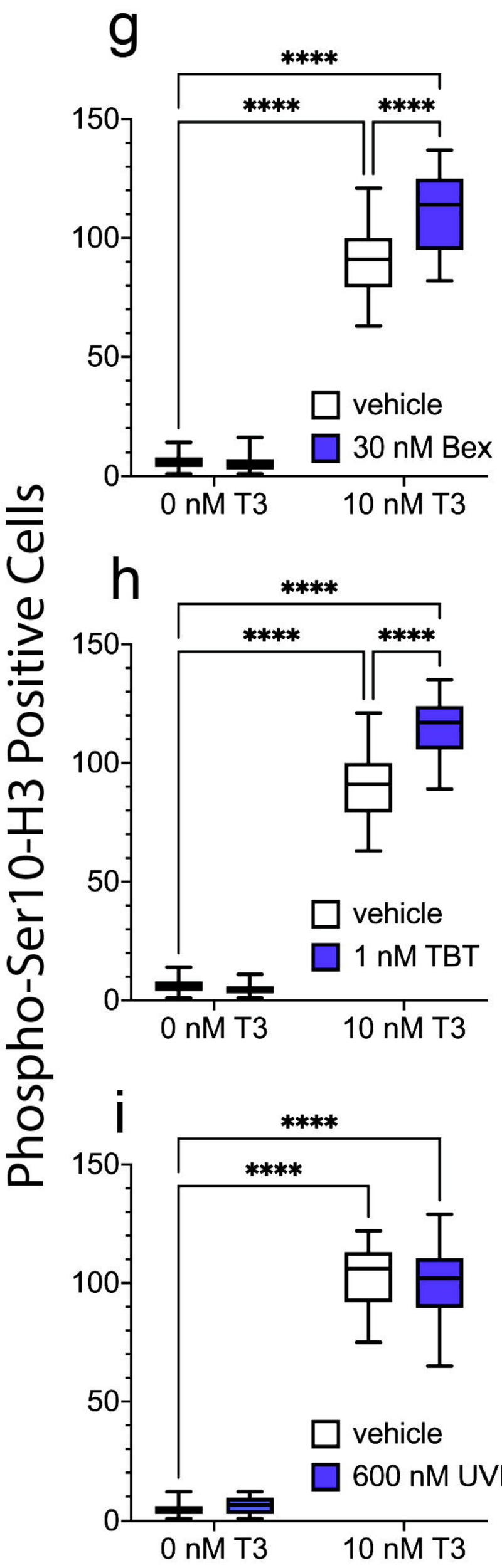


Figure 2





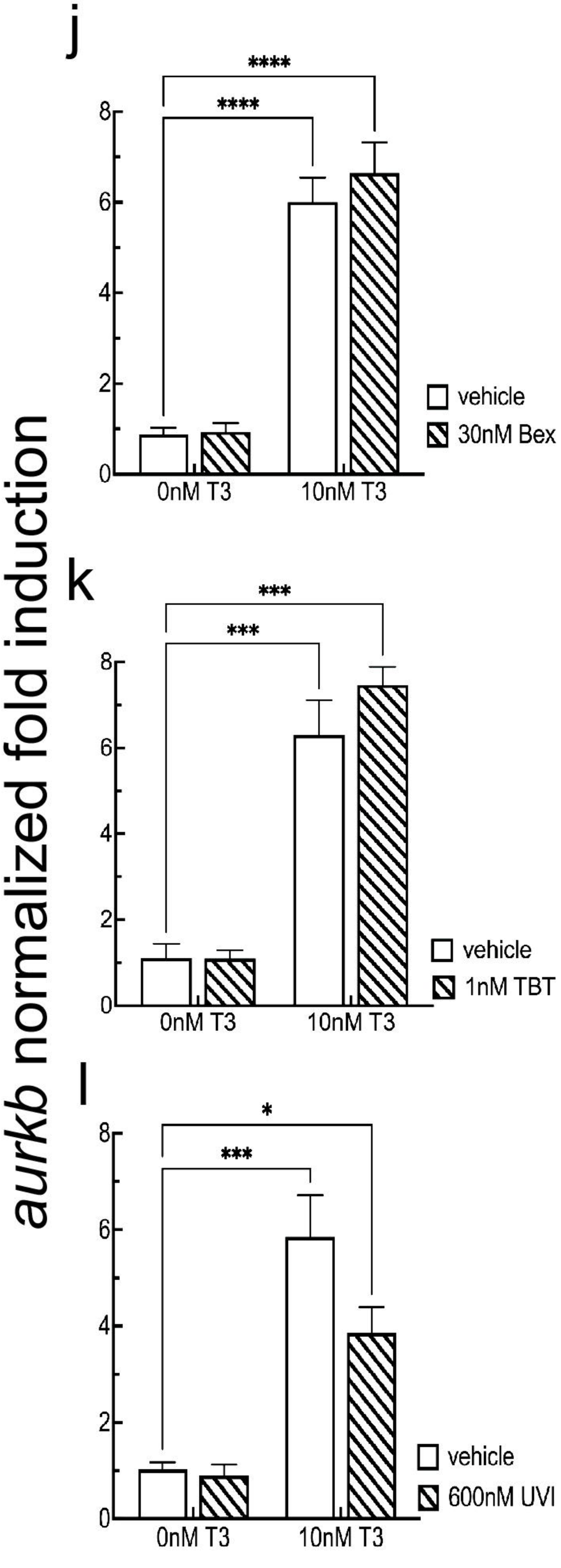


Figure 3

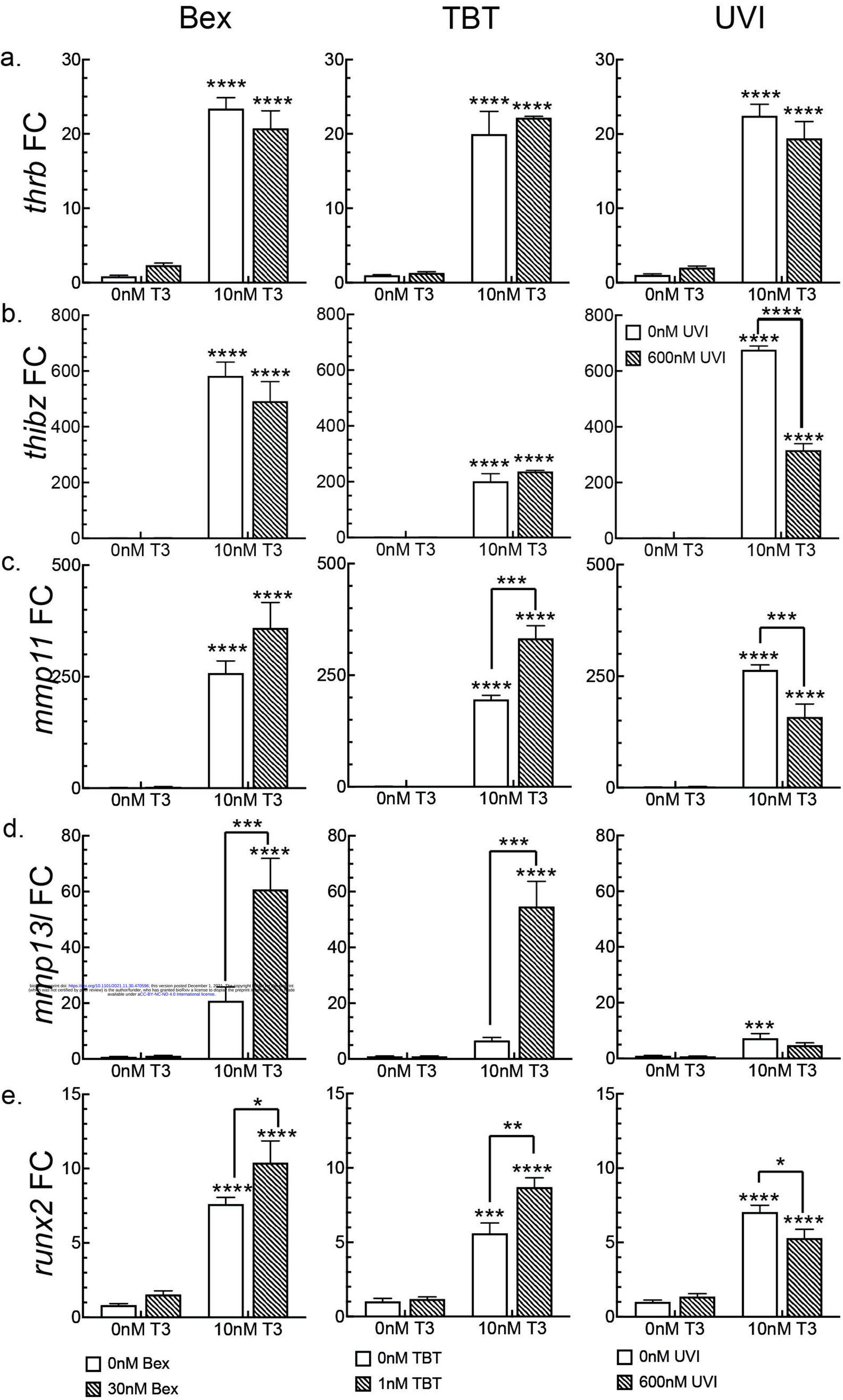
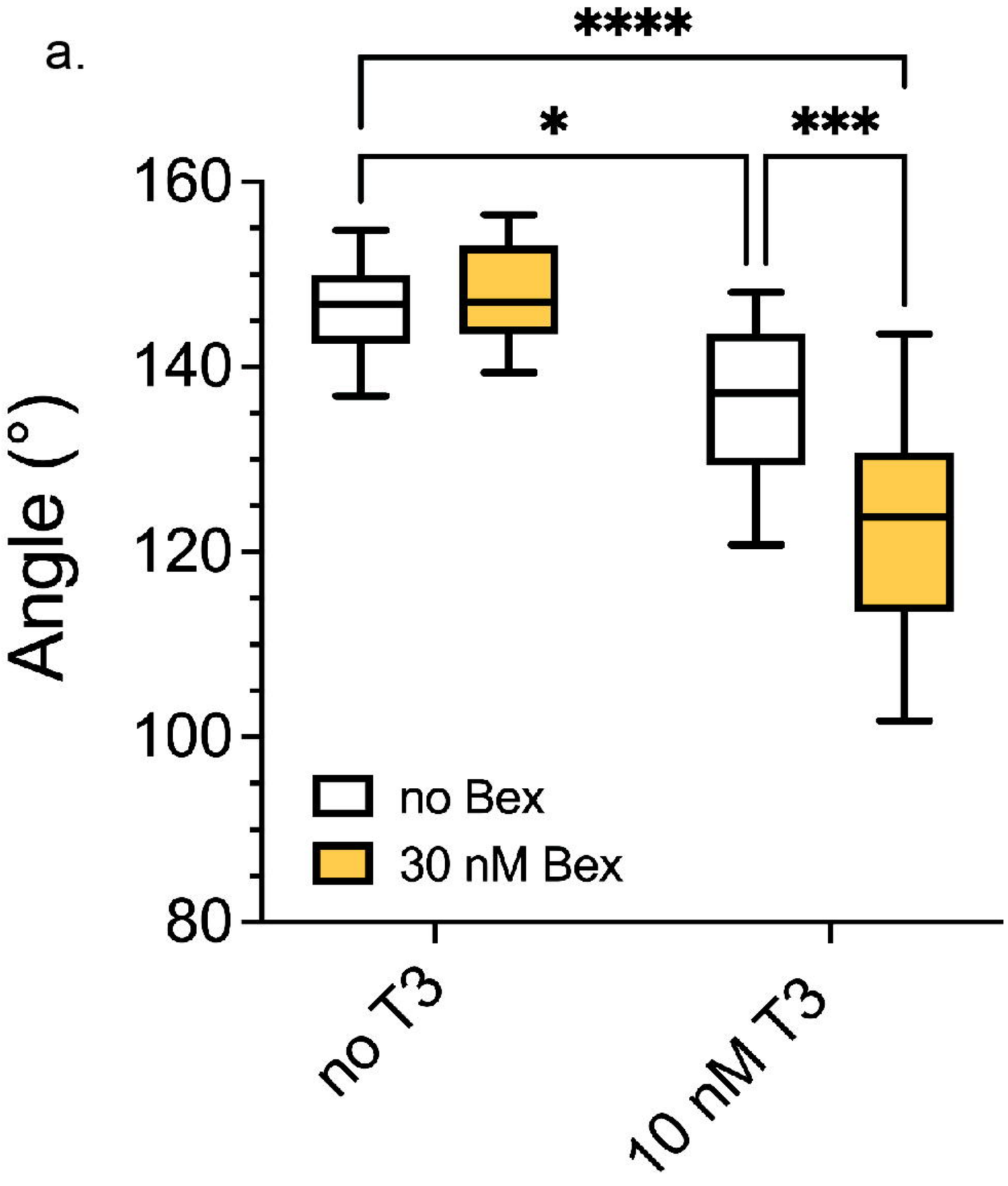
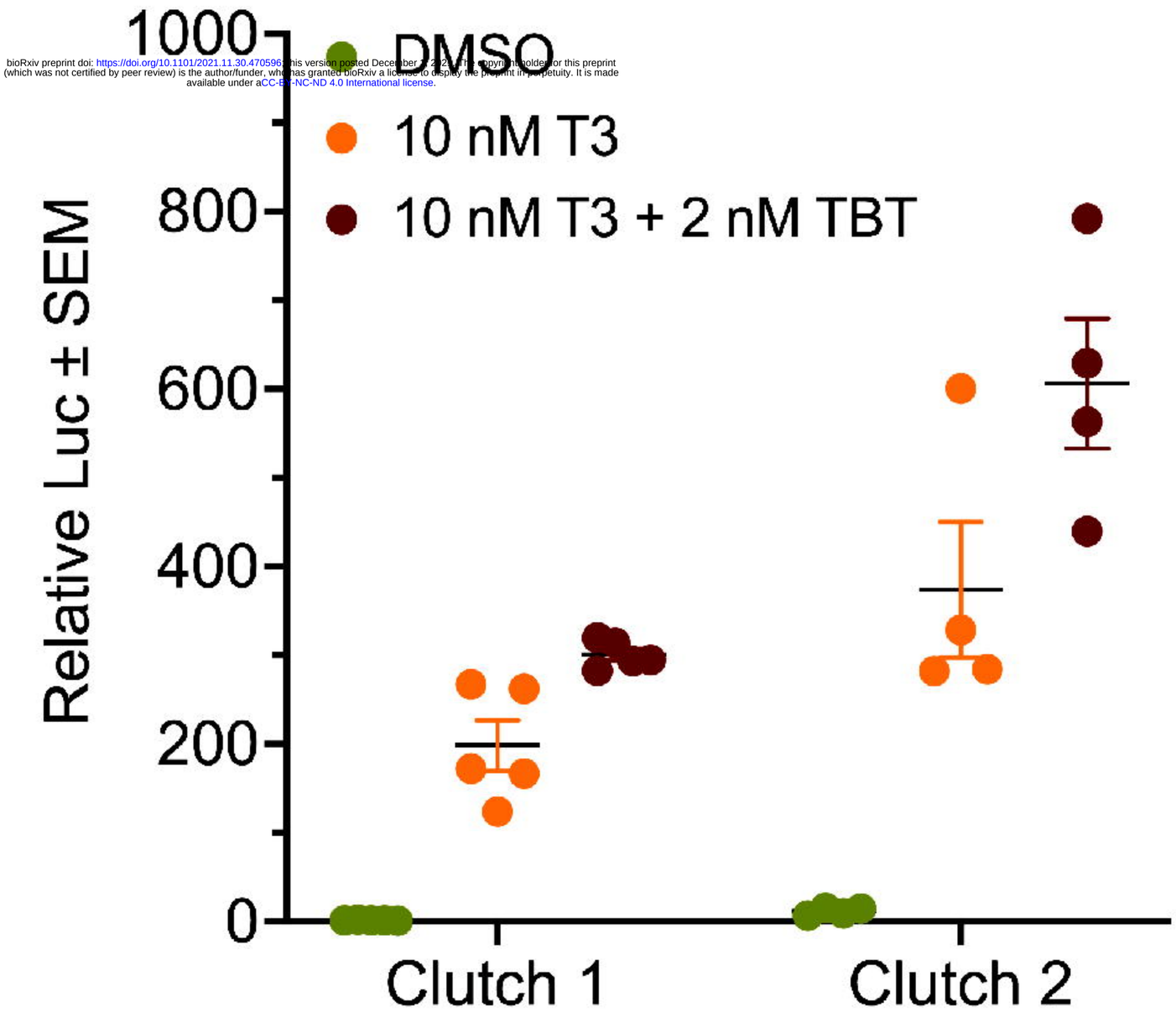


Figure 4

a.



b.



2-way ANOVA
Source of Variance
treatment: $p < 0.0001$
clutch: $p = 0.0005$

Tukey MCT
DMSO vs T3: $p < 0.0001$
DMSO vs T3+TBT: $p < 0.0001$
T3 vs T3+TBT: $p = 0.0092$