Impact of copper nanoparticles and copper ions on transcripts involved in neural repair mechanisms in rainbow trout olfactory mucosa

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Impact of copper nanoparticles and copper ions on transcripts involved in neural repair mechanisms in rainbow trout olfactory mucosa

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Running head: Effect of nanocopper on repair pathway in olfactory mucosa

Abstract

Olfactory mucosa is well-known for its lifelong ability for regeneration. Regeneration of neurons and regrowth of severed axons are the most common neural repair mechanisms in olfactory mucosa. Nonetheless, exposure to neurotoxic contaminants, such as copper nanoparticles (CuNPs) and copper ions (Cu^{2+}), may alter the reparative capacity of olfactory mucosa. Here, using RNA-sequencing, we investigated the molecular basis of neural repair mechanisms that were affected by CuNPs and Cu^{2+} in rainbow trout olfactory mucosa. The transcript profile of olfactory mucosa suggested that regeneration of neurons was inhibited by CuNPs. Exposure to CuNPs reduced the transcript abundances of pro-inflammatory proteins which are required to initiate neuroregeneration. Moreover, the transcript of genes encoding regeneration promoters, including canonical Wnt/β-catenin signaling proteins and developmental transcription factors, were downregulated in the CuNP-treated fish. The mRNA levels of genes regulating axonal regrowth, including the growth-promoting signals secreted from olfactory ensheathing cells, were mainly increased in the CuNP treatment. However, the reduced transcript abundances of a few cell adhesion molecules and neural polarity genes may restrict axonogenesis in the CuNP-exposed olfactory mucosa. In the Cu^{2+}-treated olfactory mucosa, both neural repair strategies were initiated at the transcript level. The stimulation of repair mechanisms can lead to the recovery of Cu^{2+}-induced olfactory dysfunction. These results indicated CuNPs and Cu^{2+} were differentially affected the neural repair mechanism in olfactory mucosa. Exposure to CuNP had greater effects on olfactory repair mechanisms relative to Cu^{2+} and dysregulated the transcripts associated with stem cell proliferation and neural reconstitution.

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Introduction

Perception of chemosensory signals conveys information to fish that is essential for survival and reproduction success. Detecting the presence of an appropriate mate, the location of food, the risk of predation, and the presence of potentially toxic contaminants in fish habitat is mediated through olfactory sensory neurons (OSNs). These OSNs receive sensory inputs and transmit them to brain for processing (Kermen et al., 2013; Laberge and Hara, 2001). Due to direct contact of OSNs with myriad biotic and abiotic environmental stressors, they are susceptible to functional impairment. Vertebrate olfactory neuroepithelium is capable of regeneration in response to damage. Reconstitution of damaged OSNs is a key process to restore olfactory function. Nevertheless, some anthropogenic contaminants can interfere with olfactory neuroepithelial regenerative capability, and consequently inhibit the neuroepithelium reestablishment (Szymkowicz et al., 2019; Wang et al., 2017a).

Copper nanoparticles (CuNPs) are emerging environmental contaminants of concern (Malhotra et al., 2020). Due to unique conductivity, catalytic, and antimicrobial properties of engineered CuNPs, they appear as a promising material to be applied in electronics, biomedical, and agriculture (Tabesh et al., 2018; Vanti et al., 2020; Zhou et al., 2019). The application of CuNPs as an antibiofouling agent in fish-cage netting (Ashraf et al., 2017), an antibiofilm agent against fish pathogens (Chari et al., 2017), and a dietary supplement to boost fish growth and immune system (El Basuini et al., 2017), make them popular in the aquaculture industry. The progressive production and utilization of CuNPs enhances their incidental input to water bodies. We have previously demonstrated that CuNPs can be taken up by rainbow trout olfactory mucosal cells and induce olfactory toxicity (Razmara et al., 2021). Exposure to CuNPs can not only disrupt OSN function (Razmara et al., 2021; Razmara et al., 2019), but also reduce the transcription of

neuroregeneration-related pathways in rainbow trout olfactory mucosa (Razmara et al., 2021). The CuNP-induced olfactory impairment was persisted after 7 days of transition to clean water, and the OSNs function did not restore (Razmara and Pyle, 2022). We also compared the toxicity of CuNP and copper ion (Cu^{2+}) which is a well-known olfactory disrupter. In Cu^{2+}-treated fish, unlike those in the CuNP treatment, neuroregenerative pathways was upregulated (Razmara et al., 2021), and the olfactory function was recovered (Razmara and Pyle, 2022). These findings indicates that CuNPs and Cu^{2+} have differential effects on the neuroregeneration process in the olfactory mucosa. Nonetheless, the detailed regenerative molecular events that were affected by each of the Cu contaminants remain unknown.

In addition to OSNs, there are other types of cells residing in the fish olfactory mucosa (Fig. 1). Mucus-secreting goblet cells and sustentacular cells (a.k.a., supporting cells) provide protection and structural functional support to the epithelial cells, respectively (Bols et al., 2001; Hegg et al., 2009). Basal stem cells are involved in regeneration processes of epithelial cells. Two types of neural repair mechanisms have been reported in a damaged olfactory mucosa. First is the genesis of new OSNs from the basal stem cells (Graziadei and Graziadei, 1979). Olfactory neural replenishment is a complex multiphase process that entails progenitors forming from olfactory stem cells followed by migration and differentiation, which ultimately gives rise to mature OSNs (Nicolay et al., 2006). The basal layer of olfactory epithelium is composed of two populations of stem cells, horizontal basal cells (HBCs) and globose basal cells (GBCs) (Fig. 1) (Roy et al., 2013). In general, GBCs are mitotically active, whereas the HBCs are a reserve population which remains mitotically quiescent under conditions of non-extensive injury (Choi and Goldstein, 2018). To maintain the integrity of olfactory neuroepithelium, both classes of stems cells are actively interacting with extrinsic regulators (e.g., immune cells) in their niche (Chen et al., 2019). These

intercellular interactions lead to activation of intrinsic regulators, such as transcription factors, that control stem cell proliferation, differentiation, and neuron maturation. The second type of repair is axonal regrowth in existing OSNs. During axon regeneration a specialized type of glial cell, olfactory ensheathing cell (OEC), secretes extrinsic growth and guidance cues and subsequently, reactivates the intrinsic developmental process in the growing axon (Roet and Verhaagen, 2014). In this study, using the transcription profile of rainbow trout olfactory mucosa, we investigated how exposure to CuNPs and Cu^{2+} differentially affected intrinsic and extrinsic regeneration regulators.

Materials and Methods

Fish husbandry and experimental design

Juvenile rainbow trout with an average weight of 26.5 ± 5.2 g (mean ± SD; n = 15) were obtained from Sam Livingston Fish Hatchery (Alberta, Canada) and housed in a holding tank (16 h light: 8 h dark photoperiod, 12 ºC) at the University of Lethbridge Aquatic Research Facility (ARF). According to our previous study, the 50% olfactory inhibitory concentrations of CuNPs and Cu^{2+} (24-h IC50 measured by electro-olfactography) were 320 ± 13 and 7 ± 1 µg/L (mean ± SD), respectively (Razmara et al., 2019). These IC50s of Cu contaminants were used as a functional unit of toxicity. Following a two-week acclimation period, to investigate the comparative effect of CuNPs and Cu^{2+} on the transcript profiles of rainbow trout olfactory mucosa, fish were exposed to 24-h IC50s of Cu contaminant for 96 h (Razmara et al., 2021). Every 24 h, fish were transferred to new sets of tanks containing fresh water and Cu solutions. Quality of culture water was measured as follows (mean ± SD; n = 3): temperature, 12.4 ± 0.3 °C; dissolved oxygen, 8.8 ± 0.7 mg/L; conductivity, 331.3 ± 0.3 µS /cm; hardness, 156 ± 3.1 mg/L as $CaCO_3$;

112 alkalinity: 125.0 ± 4.4 mg/L as $CaCO_3$; median pH, 8.1 (range 7.8 – 8.4), and dissolved organic
113 carbon, 2.5 ± 0.7 mg/L. During the exposure period fish were not fed.

114 **Preparation of Cu stock mixtures, Cu analysis, and CuNPs characterization**

115 Copper solution preparation and analysis were described by Razmara et al., 2021. In short,
116 using a 30 min water bath sonicator (UD 150SH6LQ; 150 W; Eumax; USA), nanocopper powder
117 (35 nm; 99.8% purity; partially passivated, Nanostructured & Amorphous Materials Inc, USA)
118 was suspended in ddH_2O (18.2 MΩ/cm water, Millipore, USA) to make a stock suspension of 250
119 mg/L CuNPs. A fresh stock solution of 100 mg/L $CuSO_4$ (>98% purity; BHD, USA) in ddH_2O
120 was prepared and used as the source of Cu^{2+}. The actual concentration of Cu in the fish tanks was
121 measured by graphite furnace atomic absorption spectrometry (240FS GFAAS, Agilent
122 Technologies, USA). The actual Cu^{2+} and CuNPs concentrations 24 h after spiking the fish tanks
123 with Cu mixtures, were 7 ± 1 μg/L and 210 ± 13 μg/L (65% of nominal CuNPs concentration),
124 respectively (mean ± SD, n = 6) (Razmara et al., 2021). The Cu concentration in the control was
125 below the GFAAS detection limit.

126 We previously characterized the CuNPs (Razmara et al., 2019), and we used the same batch
127 nanocopper powder for this study. The CuNPs were semi-spherical with an average diameter of
128 32 ± 1 nm (mean ± SEM). Polydispersity index (PDI), hydrodynamic diameter (HDD), and zeta
129 potential, which are indicators of NP aggregation, were changed over 24 h. Sixteen h after spiking
130 the fish tanks water with CuNPs, we observed a significant increase in polydispersity (PDI = 1)
131 and HDD, along with a noticeable reduction in zeta potential. These measurements indicated
132 CuNPs were less stable and consequently, less bioavailable over the last few hours of exposure
133 (Razmara et al., 2019). Using Amicon Ultra-4 Centrifugal Filter Units (3K regenerated cellulose
134 membrane, Merck Millipore Ltd., USA), dissolved Cu was separated from CuNPs. The

concentration of dissolved Cu in the filtrate was measured using GFAAS over 24 h (Razmara et al., 2019). We observed a gradual release of dissolved Cu in the fish tanks over time (the highest dissolution after 24 h was 14 µg/L) (Razmara et al., 2019). Therefore, the effects of CuNPs on olfactory mucosa is associated to both nanocopper and dissolved Cu.

RNA isolation

As described in Razmara et al. (2021), following the 96-h exposure to CuNPs and Cu^{2+}, fish (n = 5) were euthanized using a pH buffered MS222 solution (240 mg/L tricaine methane sulfonate (AquaLife, Canada) and 720 mg/L $NaHCO_3$ (Fisher Scientific, USA)). Immediately after euthanizing, fish olfactory rosettes were dissected, flash-frozen in liquid nitrogen, and stored in -80°C. Using RNeasy Mini Kit manufacturer's protocol (catalogue # 74106; Qiagen), total RNA was isolated from olfactory rosettes. Quality and quantity of isolated RNA were determined by a Nanodrop spectrophotometer (Thermo Scientific NanoDrop One spectrophotometer, Thermo Scientific). The RNA integrity number (RIN) was determined by Bioanalyzer 2100 (Agilent Technologies). The RIN of all samples were > 7 (Razmara et al., 2021).

Illumina sequencing and RNA-seq analysis

High quality RNA samples were shipped to Canada's Michael Smith Genome Sciences Centre (GSC, BC cancer research, Canada) to construct strand specific mRNA libraries. Each library was sequenced using the HiSeq 2500 (Illuina, San Diego, California, USA) as paired-end platform generating 2 * 75 base pair (bp) reads for each sample (Razmara et al., 2021). The RNA-seq analysis pipeline is fully described in Razmara et al. (2021). In brief, reads were assessed for their quality using FastQC (Andrews, 2020), and high quality reads were aligned to rainbow trout reference transcriptome (Omyk_1.0, GCF_002163495.1) using STAR two-pass alignment version 2.6.1 (Dobin et al., 2013). Using StringTie version 1.3.4, mapped reads were assembled and

158 counted (Pertea et al., 2015). The counted transcripts were annotated against National Center for
159 Biotechnology Information (NCBI) non-redundant (nr) protein database using BLASTx.
160 Differential gene expression analyses was conducted using DESeq2 version 1.28.1 (Love et al.,
161 2014) with a $p_{adj} \leq 0.05$ cut-off for statistical significance. Of the total number of transcripts in
162 Cu^{2+} (53141) and CuNPs (53380) treatments, 2.1% were differentially expressed in each treatment.
163 However, there was a narrow overlap between the CuNP- and Cu^{2+}- affected transcripts (Razmara
164 et al., 2021). The functional annotations of the differentially expressed transcripts was performed
165 in Blast2Go Pro (OmicsBox version 1.3.11) (Götz et al., 2008). The functional annotation analysis
166 showed 87% and 89% of the differentially expressed transcripts were functionally annotated in
167 CuNPs and Cu^{2+} treatment, respectively (Razmara et al., 2021). Enrichment analysis of gene
168 ontology (GO) terms was conducted using the Fisher's exact test in Blast2GO ($p \leq 0.05$).

169 **Gene expression quantification by qPCR**

170 The results of RNA-seq were previously validated and confirmed by qPCR gene expression
171 (Razmara et al., 2021). In this study, the expression *TCF7L2*, a key transcription factor involved
172 in Wnt signaling pathway, was studied in both Cu treatments using qPCR. One µg of the total
173 RNA was used to make cDNA using (QuantiTec Reverse Transcription Kit, catalogue # 205311,
174 Qiagen). The TCF7L2 primer set was designed using Primer-Blast online software provided by
175 NCBI (Ye et al., 2012). To ensure the specificity of the primers' amplification, the PCR products
176 were purified (QIAquick PCR Purification kit, catalogue # 28104; Qiagen), sequenced, and blasted
177 in NCBI. The primer sequences for *TCF7L2* (primer efficiency: 102%) were as follows: F -
178 CGAATCAAAGTCCGAACGCA, R - TTTGGAAACGCCGTCGAGA. The quantification of
179 gene expression analysis was carried out in duplicate on a Bio-Rad thermocycler (C1000 Touch
180 thermocycler, CFX96 Real-Time System, Bio-Rad) using RT² SYBR Green qPCR master mix

(catalogue # 330503, Qiagen) as described by Razmara et al. (2021). Expression of *TCF7L2* was normalized to the expression of reference genes, *ACTB* (Aegerter et al., 2005) and *EF1a* (Polakof et al., 2010), which did not changed in the transcriptome analysis. Quantification of the *TCF7L2* transcripts was analyzed according the Pfaffl method of relative quantification (Pfaffl, 2001). To determine whether *TCF7L2* was differentially expressed in response to (CuNPs and Cu^{2+}, we conducted one-way analysis of variance (ANOVA) with Tukey's post hoc test using R, version 3.6 (R Core Team, 2019).

Results and discussion

Effect CuNPs and Cu^{2+} on the repair mechanism in the olfactory mucosa

Results of pathway enrichment analysis indicated a number of neuroregeneration-related functional GO terms, including neurogenesis, were composed of downregulated genes in the CuNP treatment (Fig. 2A). We previously reported that CuNPs, but not Cu^{2+}, were significantly accumulated in the olfactory mucosal cells (Razmara et al., 2021). In the CuNP-treated fish, there was a progressive impairment in the olfactory responses over time (Razmara et al., 2019). In consequent of the dysregulated repair mechanisms, the neurophysiological responses of CuNP-exposed OSNs remained impaired after the 7 days of recovery period (Razmara and Pyle, 2022). These results indicate CuNPs had adverse effects on the OSNs which may not be simple to repair. Our current knowledge of possible effects of CuNPs on olfactory neuroregeneration is scarce. In the central nervous system of zebrafish embryos, exposure to copper oxide nanoparticles resulted in reduced neural development (Xu et al., 2017).

On the other hand, the upregulation of genes encoding developmental-related pathways in the Cu^{2+} treatment, such as cell cycle and epithelium development (Fig. 2B), reflects that regeneration processes were activated in the olfactory mucosa. Despite a continuous exposure to

204 Cu^{2+}, we previously observed a partial recovery in Cu^{2+}-exposed OSNs function (Razmara et al.,
205 2019). After 7 days of exposure to Cu^{2+} the fish olfactory function was fully recovered (Razmara
206 and Pyle, 2022). This functional olfactory recovery was a further indication of active repair
207 mechanisms in the Cu^{2+} treatment. A 24-h exposure of the olfactory system to Cu^{2+} increased the
208 level of neurogenesis-related miRNAs and mRNAs in zebrafish (Tilton et al., 2008; Wang et al.,
209 2013). Consistent with our results, developmental pathways were enriched in the Cu^{2+}-exposed
210 zebrafish olfactory system (Tilton et al., 2008). A recent study indicated that different classes of
211 OSNs (i.e. ciliated and microvillous OSNs) in zebrafish larvae display distinct reparative responses
212 following a 24-h exposure to 16 – 635 µg/L Cu^{2+} (Ma et al., 2018). Regeneration of microvillous
213 cells occurred following a 72-h recovery period whereas, there was no significant regeneration of
214 ciliated OSNs in the Cu^{2+}- exposed larvae. Nonetheless, ciliated cells showed a partial functional
215 improvement following the recovery period (Ma et al., 2018). These results suggested that in the
216 ciliated OSNs, rather than replacement of the injured OSNs with new neurons, axonal regeneration
217 was activated to repair the Cu^{2+}-induced injuries. Confocal images of Cu^{2+}-exposed zebrafish
218 demonstrated that ciliated OSNs had more axon retractions than microvillous cells (Ma et al.,
219 2018). Axonal retractions can stimulate axonal repair mechanisms in ciliated cells. In our study,
220 the upregulation of genes involved in the cell projection GO term suggests axonogenesis may be
221 activated in both CuNP and Cu^{2+} treatment (Fig. 2).

222 **Inflammatory responses in the CuNP- and Cu^{2+}-exposed olfactory mucosa**

223 In addition to the defensive role of inflammatory response pathway in the olfactory
224 neuroepithelium, inflammation contributed towards the regulation of regeneration (Chang and
225 Glezer, 2018; Chen et al., 2017, 2019; Crisafulli et al., 2018). The results of pathway enrichment
226 analysis showed that genes encoding for proinflammatory chemokines and cytokine signaling were

significantly downregulated in the CuNP-treated olfactory mucosa (Fig. 2A). The reduced transcript abundances of many pro-inflammatory genes including *IL-1β*, *CCL4*, *CCL19*, *CCL20*, and *CCL25* (Table 1), in the CuNP treatment suggest CuNPs may act as an anti-inflammatory treatment in the rainbow trout olfactory mucosa. Previous studies demonstrated a number of metal NPs, including CuNPs, can have anti-inflammatory properties (Agarwal et al., 2019; Thiruvengadam et al., 2019). Blocking of cytokine signaling is reported as the primary mechanism of anti-inflammation induced by metal NPs (Agarwal et al., 2019).

Acute inflammation plays a positive role in neural stem cells function. In the adult zebrafish brain, acute inflammation is required to activate neuroregeneration, and administration of ani-inflammatory dexamethasone (Dex) impeded the regeneration of lesioned brain (Kyritsis et al., 2012). In the mouse olfactory mucosa, repair of lesioned neuroepithelium was also reliant on the induction of acute inflammation (Chen et al., 2017). In Dex-treated mice with low transcript abundances of proinflammatory cytokines, the proliferation and subsequent differentiation of HBCs were significantly impaired (Chen et al., 2017). Moreover, a three-day treatment of lesioned olfactory mucosa with Dex resulted in a marked reduction in the number of regenerated OSNs in mice (Crisafulli et al., 2018). The potential anti-inflammatory properties of CuNP may block stem cell proliferation and neuroregeneration in the olfactory neuroepithelium which needs further investigation.

In addition to the low transcript level of *IL-1β* cytokine in the CuNP treatment, activation of its protein may be impaired by CuNPs. The pro IL-1β needs to be activated by a protein complex known as inflammasome (Liu et al., 2017). The transcript abundances of key components of inflammasomes, including *CASP1*, *NLRP3*, and *MEFV* (encoding pyrin) — which are necessary to cleave the pro IL-1β (Liu et al., 2017; Sharma et al., 2019) — was reduced (Table 1). Previous

studies have demonstrated that transient inflammation mediated by IL-1β promotes regeneration in zebrafish (Hasegawa et al., 2017; Tsarouchas et al., 2018). Inhibiting pro IL-1β activation by blocking CASP1 activity resulted in weak axonal regeneration in zebrafish spinal cord (Tsarouchas et al., 2018). Moreover, IL-1β has been reported to promote the olfactory stem cells migration in a concentration-dependent manner in rat neuroepithelium (Pu et al., 2018). Hence, the downregulation of *IL-1β* and its activators by CuNPs may inhibit olfactory neuroregeneration.

One of the primary cellular inflammatory pathways is nuclear factor-κB (NF- κB) signaling, which regulates expression of a large array of genes implicated in inflammation including *IL-1β* and *NLRP3*. The NF- κB transcription factors mediate two signaling pathways, non-canonical (a.k.a., NIK/NF- κB signaling) and canonical pathways, which both regulate cellular inflammation despite applying different signalling mechanisms (Liu et al., 2017). The NF-κB transcription factor family composed of five members, including NF-κB1 (P50), NF-κB2 (P52), RELA(P65), RELB, and c-REL which are operating as dimeric complexes activating κB enhancer (Pasparakis, 2009). Pathway enrichment analysis showed the NIK/NF- κB signaling was over-represented in the CuNP treatment (Fig. 2). In the NIK/NF- κB signaling pathway, association of specific ligands with tumor necrosis factor superfamily (TNFR), such as CD40, stimulates the production of mature P52 from its precursor p100 (Sun, 2011). Processing of p100 is mediated by NF- κB-inducing kinase (NIK) which activates an inhibitor of NF- κB kinase (IKK) to subsequently induce phosphorylation and ubiquitination of p100 and generate mature p52 (Sun, 2017). The mature p52 forms a heterodimer complex with RELB and, together, translocate to the nucleus to induce the transcription of target genes, such as *CCL19* (Valiño-Rivas et al., 2016). Figure 3A displays that exposure to CuNPs reduced the transcript abundances of *CD40*, *P52* in the NIK/NF- κB signaling pathway. The canonical NF- κB pathway was also affected by CuNPs (Fig.

3B). In the canonical NF- κB signaling, different stimuli including cytokines and growth factors activate the IKK to phosphorylate the inhibitor of NF- κB (IκBα) and trigger its degradation. The degradation of IκBα results in nuclear translocation of NF- κB transcription factor dimers (P50/RELA or P50/c-REL) and initiation of target gene transcriptions (e.g., *IL-1β*, *NLRP3*, *CCL4*, *CCL20*). In the CuNP-treated olfactory mucosa, while the mRNA level of *c-REL* was reduced, the expression of *IκBα* was slightly increased (Table 1 and Fig. 3B). The NF-κB signaling pathway plays a critical role in early stages of neurogenesis. Differentiation of neural stem cells is reliant on the activation of NF-κB signaling, and inhibition of canonical NF-κB signaling can block asymmetric cell division which is essential for proper neurogenesis (Zhang et al., 2012). Moreover, impairment of the canonical NF-κB pathway through deletion of RelA inhibited the neuroregeneration in HBCs (Chen et al., 2017). Impairment of NF-κB signaling pathway may comprise the acute inflammatory responses in the CuNP-treated olfactory mucosa. Given the importance of acute inflammation in neurogenesis initiation, the downregulation of transcripts associated with canonical and non-canonical NF-κB inflammatory pathways suggests neuroregeneration was inhibited in the CuNP treatment. This notion is supported by the continued olfactory impairment of CuNP-exposed fish after the 7-day recovery period.

In contrast to the CuNP treatment, gene expression of pro-inflammatory cytokines and chemokines remained unchanged in the Cu^{2+} treatment. The expression of *NLRP1b*, an important component of inflammasomes involved in CASP1 activation (de Rivero Vaccari et al., 2014), displayed a significant upregulation in Cu^{2+}-treated fish (Table 1). The activation of CASP1 can consequently enhance the pro IL-1β cleavage and induce inflammation. Furthermore, the transcript abundances of *NLR12* and *NLRC3*, that both negatively regulate the NF-κB inflammatory signaling (Gharagozloo et al., 2015; Tuncer et al., 2014), were reduced (Table 1). These data

suggest that olfactory mucosal cells' inflammatory responses were not inhibited as a result of exposure to Cu^{2+}, and consequently, neurogenesis pathways may be activated.

Effect of Cu contaminants on canonical Wnt/β-catenin signaling pathway

In addition to the suppression of inflammatory signaling, Wnt signaling, one of the major pathways regulating stem cell proliferation and differentiation, was affected by CuNPs (Fig. 2). In agreement with our results, a transcript-based study on the lungs of mice exposed to inhalation of copper oxide nanoparticles for two weeks, indicated that the Wnt signaling pathway was significantly enriched (Rossner et al., 2020). Furthermore, copper oxide nanoparticles inhibited the Wnt signaling pathway in prostate cancer cells and attenuated the stemness of cancer cells (Wang et al., 2017b).

Activation of canonical Wnt/β-catenin pathway promotes neurogenesis in the olfactory epithelium (Wang et al., 2011). In GBCs, Wnt/β-catenin pathway plays a key role in maintaining proliferation and promoting neuroregeneration (Chen et al., 2014). Moreover, the activation of Wnt/β-catenin signaling was both necessary and sufficient to switch the HBCs from a resting state to an activated neurogenic state (Fletcher et al., 2017). In the Wnt/β-catenin pathway, when the Wnt ligand is present, β-catenin relocates from cytoplasm to the nucleus, where it binds to the TCF/LEF transcription factor to promote the expression of many genes involved in cell proliferation (Bengoa-Vergniory and Kypta, 2015). Inhibition of the Wnt/β-catenin signaling through disruption of β-catenin/TCF complex transcriptional activity prevented the regeneration of injured olfactory epithelium (Wang et al., 2011). Following exposure to CuNPs, there was a considerable reduction in the expression of *TCF7L2*, which is a downstream effector of the Wnt pathway (Table 1 and Fig. 4A). The *TCF7L2* gene expression analysis by qPCR confirmed the result of RNA-seq (Fig. 4B). Of the 4 members of the TCF/LEF transcription factor family,

TCF7L2 was the most expressed transcription factor in the mouse's entire olfactory epithelium, which had a dense expression in basal stem cells (Wang et al., 2011). In zebrafish, TCF7L2 positively regulated the Wnt/β-catenin pathway in midbrain, and TCF/LEF transcription factors contributed in the homeostasis of olfactory epithelium (Shimizu et al., 2012). As a result of the *TCF7L2* downregulation, the neuroregeneration might be reduced in the CuNP-treated fish.

In the absence of Wnt, β-catenin is actively degraded by a destruction complex in the cytoplasm, which consequently diminishes the expression of target genes. One of the components of the β-catenin destruction complex is APC (a tumor suppression factor) (Fig. 4A). The APC is a multifunctional protein that not only mediates stem cell maintenance through the regulation of cytoplasm β-catenin, but also promotes neuronal differentiation. Deletion of APC in adult neural stem cells can exhaust the germinal zone and disturb differentiation and migration of neuroblasts in the olfactory bulb (Imura et al., 2010). Our results indicated a serious depletion of *APC* transcript level in the CuNP treatment (Table 1 and Fig. 4A). The reduced transcript abundances of *TCF7L2* and *APC*, which are involved in the positive and negative regulation of Wnt/β-catenin pathway, respectively, suggests that neurogenesis was disrupted in the CuNP-treated olfactory mucosa. In contrast to the CuNP treatment, there was a significant increase in the mRNA of *APC* in the Cu^{2+} treatment (Table 1). These data suggest that CuNPs and Cu^{2+} distinctly influenced neurogenesis-related processes in olfactory mucosa.

Effect of CuNPs and Cu^{2+} on transcription factors associated with olfactory neurogenesis

Transcription factors (TFs) play critical roles in olfactory neuroregeneration (Nicolay et al., 2006; Shetty et al., 2005). In each stage of neurogenesis, specific TFs are activated to mediate and regulate differentiation in the olfactory mucosa. One of the key transcription factors involved

342 in olfactory epithelium development and neuroregeneration is PAX6 (Collinson et al., 2003; Davis
343 and Reed, 1996; Suzuki et al., 2015). The expression of PAX6 was reported in proliferating GBCs
344 in normal and lesioned olfactory epithelium of adult rat (Guo et al., 2010). Deletion of PAX6 in
345 HBCs has resulted in a thinner olfactory epithelial layer with reduced numbers of OSNs in mice
346 (Suzuki et al., 2015). In our study, we observed a 6-fold reduction in the expression of *PAX6*
347 following exposure to CuNPs (Table 1). A previous study also demonstrated that the PAX6 protein
348 expression was reduced in the brain of zebrafish embryos that were exposed to copper oxide
349 nanoparticles (Xu et al., 2017). PAX6 has been shown as a downstream target of the β-catenin-
350 TCF transcriptional complex in the Wnt/β-catenin pathway; the β-catenin-TCF complex binds to
351 the PAX6 promoter and induces its expression (Gan et al., 2014). Given that the expression of
352 *TCF7L2* was severely repressed in the CuNP treatment, and the reduced expression of *PAX6,* the
353 observed reduction of neurogenesis is plausible in CuNP-exposed olfactory mucosa. Additionally,
354 the expression of *LhX2* — a transcription factor engaged in the formation of mature OSNs
355 (Berghard et al., 2012; Kolterud et al., 2004) — was also downregulated following exposure to
356 CuNPs (Table 1). Another downregulated TF in the CuNP treatment is *GATA3*, which is an
357 essential TF to initiate stem cells proliferation in adult zebrafish brain (Kizil et al., 2012). The
358 downregulated expression of neurogenesis-related TFs suggested neuroregeneration was impaired
359 in the CuNP treatment.

360 In contrast to the CuNP-exposed fish, a number of TFs that regulate neurogenesis were
361 significantly upregulated following the exposure to Cu^{2+}. For instance, the expression of both
362 *PAX6* and *LhX2* transcription factors were increased in the olfactory mucosa of the Cu^{2+}-exposed
363 fish (Table 1). Besides the *PAX6* and *LhX2*, which belong to the homeobox genes, the expression
364 of two other genes from this group, *PBX1* and *OTX1*, were also upregulated (Table 1). The

transcriptional activity of both PBX1 (Grebbin and Schulte, 2017) and OTX1 (Heron et al., 2013; Pirrone et al., 2017) regulate neuroregeneration in the olfactory mucosa. Another neurogenesis master TF is *SOX2* (Sokpor et al., 2018), which was significantly induced by Cu^{2+} (Table 1). In the absence of SOX2 in HBCs, while the formation of a neuronal lineage was inhibited, the production of non-neural (i.e., sustentacular) cells was not affected. Hence, the regeneration of OSNs relies on SOX2 activity in an injured olfactory epithelium (Gadye et al., 2017). Zinc finger transcription factor *SP8*, which is involved with the development of olfactory epithelium and the olfactory bulb (Kasberg et al., 2013; Waclaw et al., 2006), was also upregulated in the Cu^{2+} treatment (Table 1). The induced gene expression of these neurogenesis-related TFs suggested that neuroregeneration could be initiated in Cu^{2+}-treated olfactory mucosa.

Effect of CuNPs on axonogenesis in the olfactory mucosa

Axonogenesis is an important stage of neural development that ultimately builds a functional connection between new neurons and target cells. In the peripheral nervous system, axonal regeneration mediates functional recovery after injury (He and Jin, 2016). When axon regeneration is initiated, the axon outgrowth will be guided to the olfactory bulb. Following the axons' exit from the olfactory epithelium to the lamina propria, OECs wrap the axons, promote axonal projection, and guide the growth cone to the olfactory bulb (Su and He, 2010). The OECs produce many signaling cues, including growth factors and cell adhesion molecules which facilitate axon regeneration (Roet and Verhaagen, 2014). Following exposure to CuNPs, the OECs altered the olfactory lamina propria microenvironment mostly in favour of axon regeneration. In fact, the expression of neurite outgrowth promoters, which are secreted from OECs, including *SERPINE1*, *SPARC*, *ADAMTS1*, *FGFR2*, *MSLN*, and *FINC* (Lin et al., 2019; Roet et al., 2013), were upregulated (Table 1). However, the expression of two cell adhesion molecules (i.e., *NCAM1*

388 and *CDH2* (a.k.a., *N-CAD*)), which can be produced by OEC to promote the axon outgrowth
389 (Rigby et al., 2020), were significantly decreased in the CuNP-exposed fish (Table 1). These cell
390 adhesion molecules, which are expressed at the surface of OECs, facilitate the attachment of axon
391 growth cones to the migrating OECs moving toward olfactory bulb (Rigby et al., 2020). In addition
392 to the OEC-derived signaling molecules, axon development is also regulated by intrinsic signals
393 (He and Jin, 2016; Mahar and Cavalli, 2018). For instance, the expression of *NTN1* (Astic et al.,
394 2002; Lakhina et al., 2012), *EPHA4* (John et al., 2002), *β3GNT1* (Henion et al., 2005), which are
395 all involved in axonal pathfinding, was upregulated following exposure to CuNPs (Table 1). These
396 data revealed that axonogenesis regulators endeavor to repair the axonal impairment that may have
397 been induced by CuNPs.

398 Neurons are highly polarized (i.e., asymmetric) cells which usually have a single long axon.
399 Polarity signaling pathways in the axon establish asymmetry in growth cones and control the
400 direction of axon growth (Zou, 2012, 2020). Axon polarity during development and regeneration
401 is regulated by signaling molecules and intracellular mechanisms which are actively interacting to
402 mediate polarity (Arimura and Kaibuchi, 2007; Stone et al., 2010). The non-canonical Wnt/planar
403 cell polarity (PCP) pathway plays a crucial role in regulating epithelial cells' polarity and axonal
404 growth cone guidance (Zou, 2020). In the PCP pathway, the Wnt ligand will bind to its receptor
405 and induce a cascade of events that ultimately activates the JNK pathway (Winter et al., 2001;
406 Zhang et al., 2012). The JNK signaling pathway regulates the expression of axonal regeneration
407 and polarity related genes (Hirai et al., 2011; Kawasaki et al., 2018; Stone et al., 2010). The
408 expression of *JUN*, which is a TF involved in JNK pathway, was significantly downregulated in
409 the CuNP treatment (Table 1). Moreover, there is molecular crosstalk between PCP and other
410 pathways driving axonal polarity, including the PAR complex (aPKC-PAR6-Par3) (Chuykin et

411 al., 2018; Zou, 2012). The interaction of PCP and PAR will increase axonal polarity. The transcript
412 abundance of *PAR3*, which serves as one key mediator of the PAR complex, was diminished
413 following exposure to CuNPs (Table 1). These data indicated that polarity mechanisms involved
414 in axonogenesis may be impaired by CuNPs.

415 Regulating the expression of axonal sprouting and guidance genes is a key step in axon
416 regeneration (He and Jin, 2016; McIntyre et al., 2010). In this study, the upregulated extrinsic and
417 intrinsic axon regrowth and guidance genes may prime the olfactory neuronal axons to regenerate
418 following exposure to CuNPs. Nevertheless, the reduction of cell adhesion molecules (i.e., *NCAM1*
419 and *CDH2*) and polarity regulating genes (i.e., *JUN* and *PAR3*) transcript levels may limit the
420 axonal outgrowth competency in the CuNP treatment. Further investigations are required to
421 determine if axon regeneration is initiated in the CuNP treatment.

422 **Effect of Cu^{2+} on axonogenesis in the olfactory mucosa**

423 In the Cu^{2+} treatment, *MSLN* was the only altered OEC-derived gene that was significantly
424 upregulated. The MSLN is a glycoprotein that is produced by OECs to make the extracellular
425 matrix suitable for axon growth (Roet et al., 2013). Although the OEC-driven axonogenesis was
426 induced more by CuNPs than Cu^{2+}, the transcript abundances of a number of intrinsic regulators
427 of axonogenesis were increased in the Cu^{2+}-exposed olfactory mucosa. Axonal outgrowth is highly
428 dependent on intact intracellular trafficking to deliver essential cargo to the growing axon
429 (McCormick and Gupton, 2020). A multi-subunit protein, BLOC1, is an important component of
430 the cell membrane and of vesicular trafficking in the developing axon (McCormick and Gupton,
431 2020). Neurons deficient in BLOC1 demonstrated insufficient neurite outgrowth in the
432 hippocampus (Ghiani et al., 2010). The expression of BLOC1 subunit 1 (*BLOC1S1*) was
433 significantly increased in the Cu^{2+} treatment (Table 1). Additionally, two endocytic accessory

proteins, *EPS15L1* (a.k.a., EPS*15R*) and *CALM*, which participate in intracellular trafficking (Moore and Baleja, 2012), were highly transcribed in the Cu^{2+} treatment. The expression of vesicular trafficking genes, including *RAB3A* and *RAB10* which regulate synaptic vesicle cycling (Pavlos et al., 2010), were also increased in the Cu^{2+}-exposed fish.

In order to have functional neural wiring in the olfactory system, the polarity and pathfinding mediators must accurately guide the neurons' axons to the olfactory bulb. One of the axonal pathfinding genes is *ROBO2*, which was upregulated in the Cu^{2+} treatment (Table 1). Previous studies have suggested that the knockout of ROBO2 in the OSNs can lead to axonal mistargeting in the OB (Cho et al., 2007; Miyasaka et al., 2005). Moreover, the expression of *PAR3* was significantly increased in Cu^{2+}-exposed mucosal cells (Table 1). Another important polarity regulation protein in the axonal growth cone is a guanine nucleotide exchange factor named TIAM1 (Montenegro-Venegas et al., 2010). The expression of *TIAM1* was also upregulated following the Cu^{2+} exposure (Table 1). The PAR complex (specifically PAR3) has been reported to form a complex with TIAM1 and regulates neural polarization (Zhang and Macara, 2006). The gene expression of two microtubule-associated proteins involved in axon formation and polarization, MAP1A and MAP1B (Halpain and Dehmelt, 2006), were increased in the Cu^{2+} treatment (Table 1). The MAP1B plays a role in axonal elongation and neural migration (Takei et al., 2000). In addition, the interaction of MAP1B and TIAM1 is fundamental to regulating polarity in axonogenesis (Montenegro-Venegas et al., 2010). Furthermore, *TRIM46*, which organizes microtubules trafficking and orientation and, consequently, regulates polarity in axons, was upregulated in the Cu^{2+} treatment (Rao et al., 2017). This upregulation of of axonogenesis-related gene transcripts in the Cu^{2+}-exposed olfactory mucosa revealed that the intrinsic neural mechanisms were in play to fulfill the axonal outgrowth requirements in the Cu^{2+}-treated fish.

Conclusions

This study provides insight into impacts of CuNPs and Cu^{2+} on the neural repair mechanisms in rainbow trout olfactory mucosa. The transcript profile of olfactory mucosa indicated that CuNPs had more inhibitory influences on neuroregeneration mechanisms relative to axonal repair mechanisms. The transcripts of many extrinsic and intrinsic neurogenesis regulators were reduced by CuNPs. The inhibition of neuroregeneration reduces the likelihood of olfactory recovery in CuNP-exposed fish. Nonetheless, exposure to CuNPs induced molecular responses that mostly promote axon regeneration in olfactory mucosa. The functionality of axonal repair in the CuNP treatment needs to be investigated further. In the Cu^{2+} treatment, the upregulated transcripts of many genes directing neurogenesis and axonogenesis reflects that both reparative mechanisms were activated. These results can explain the previously observed functional recovery of Cu^{2+}-exposed OSNs over the 96-h exposure period in rainbow trout. Given that CuNPs may impair the OSN function and reconstitution, development of a water quality criterion to protect fish against CuNPs is warranted.

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Statements and Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Both authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Parastoo Razmara. The first draft of the manuscript was written by Parastoo Razmara, and both authors commented on previous versions of the manuscript. Both authors read and approved the final manuscript

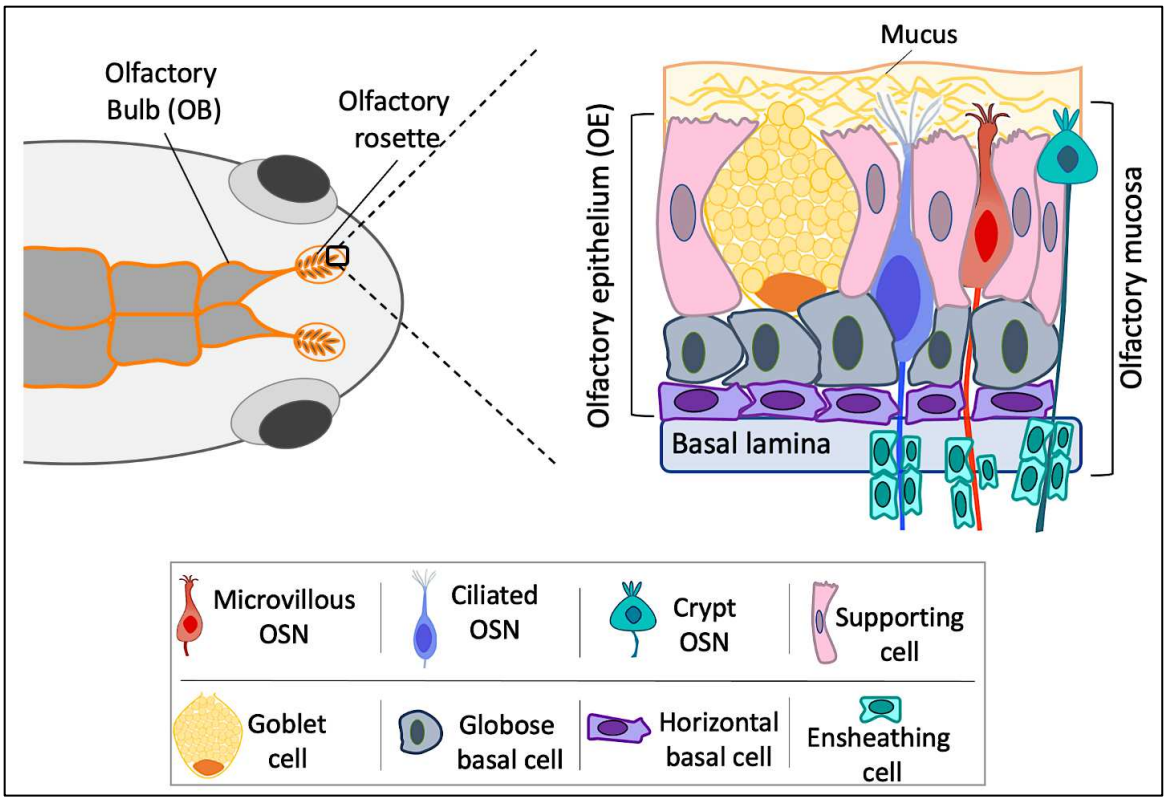
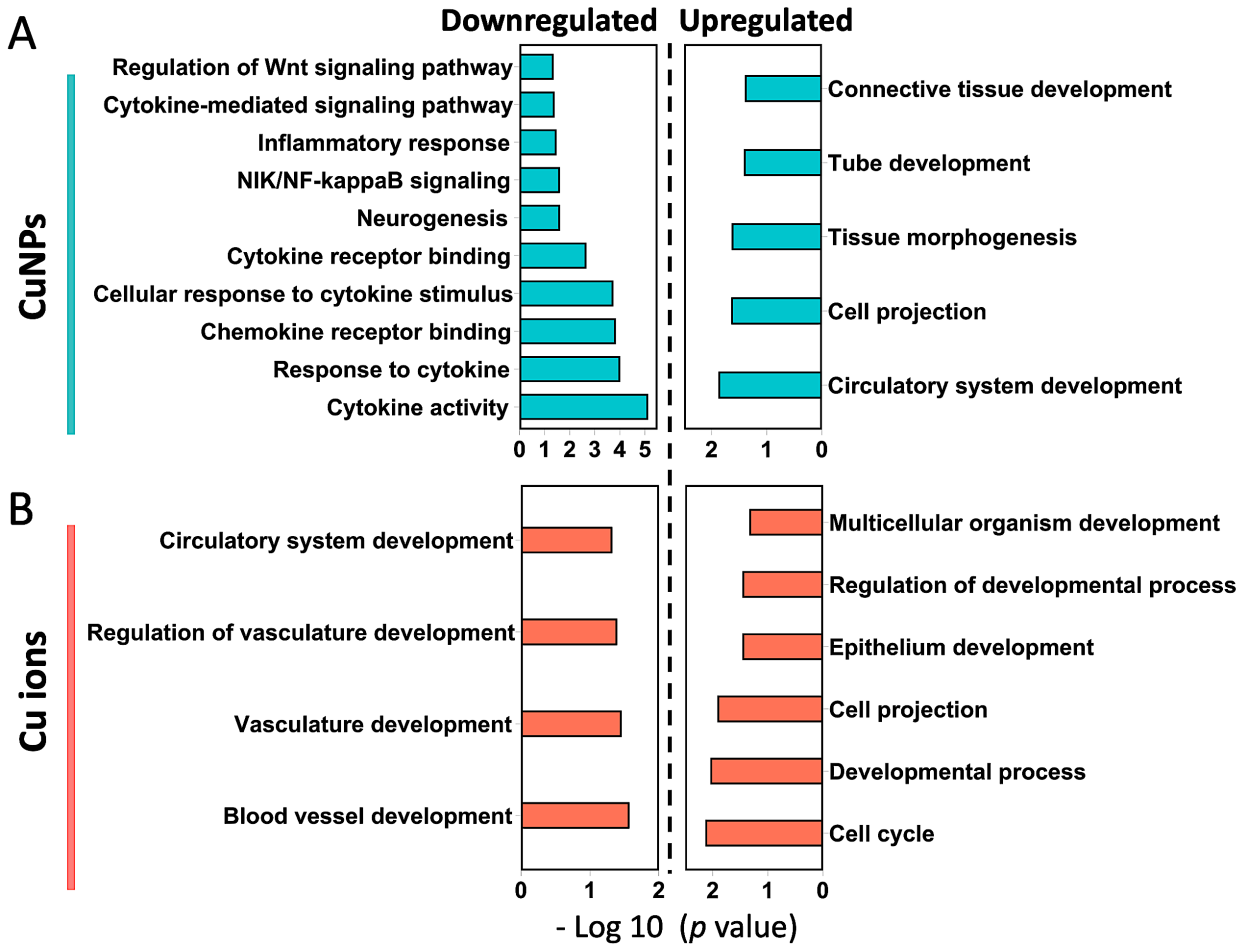


Figure 1. Schematic representation of fish olfactory system. Left drawing shows the position of olfactory system in the dorsal anterior side of the head. Right drawing shows the organization of different types of sensory (i.e., olfactory sensory neurons (OSNs)) and non-sensory cells residing in the olfactory mucosa.



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796 Figure 2. Over-represented functional GO terms associated with regeneration in CuNP- and

797 Cu^{2+}-treated rainbow trout olfactory mucosa. Bar graphs show the enriched GO terms of genes

798 that were significantly upregulated or downregulated in CuNPs (A) and Cu^{2+} (B) treatment. The

799 GO terms ordered according to -Log10 (*p* value). The p value for each GO term was calculated

800 through Fisher's exact test ($p \leq 0.05$).

801 Table 1. List of differentially expressed genes that regulate repair mechanisms in the rainbow
802 trout olfactory mucosa following exposure to CuNPs or Cu^{2+}. Gene expression was analysed by
803 RNA-seq. * indicates significant expression relative to the control and "Inf" indicates > 150-fold
804 change
805

Biological process	Gene name	Fold change in CuNPs	Fold change in Cu^{2+}
Inflammatory response	Interleukin-1 beta (*IL-1β*)	0.3 *	0.9
	C-C motif chemokine 4 (*CCL4*)	0.3 *	0.5
	C-C motif chemokine 19 (*CCL19*)	0.4 *	0.8
	C-C motif chemokine 20 (*CCL20*)	0.2 *	0.7
	C-C motif chemokine 25 (*CCL25*)	0.6 *	1
	Caspase-1 (*CASP1*)	0.7 *	0.9
	NACHT, LRR and PYD domains-containing protein 3 (*NLRP3*)	0.8 *	1
	Pyrin (*MEFV*)	0.2 *	1
	Proto-oncogene c-Rel (*c-REL*)	0.6 *	1
	NF-kappa-B inhibitor alpha (*IκBα*)	1.2 *	0.9
	NF-kappa-B p100 subunit (*NF-κB2* or *P52*)	0.8 *	1.1

	Tumor necrosis factor receptor superfamily member 5 (*TNR5* or *CD40*)	0.7 *	1
	NACHT, LRR and PYD domains-containing protein 1b (*NLRP1b*)	0.9	5 *
	NACHT, LRR and PYD domains-containing protein 12 (*NLRP12*)	1.2	0.5 *
	NLR family CARD domain containing 3 (*NLRC3*)	0.8	0.5 *
Wnt/β-catenin signaling	Transcription factor 7-like 2 (*TCF7L2*)	0 *	2
	Adenomatous polyposis coli protein (*APC*)	0 *	10.2 *
Neurogenesis	Paired box protein Pax-6 (*PAX6*)	0.2 *	1.6 *
	LIM/homeobox protein Lhx2 (*LHX2*)	0.7 *	1.7 *
	GATA-binding factor 3 (*GATA3*)	0.6 *	0.9
	Pre-B-cell leukemia transcription factor 1 (*PBX1*)	1.4	4.1 *
	Homeobox protein OTX1 (*OTX1*)	0.7	1.4 *
	Transcription factor SOX-2 (*SOX2*)	0.9	1.3 *
	Transcription factor SP8 (*SP8*)	1	1.4 *
Axonogenesis	Serpin family E member 1 (*SERPINE1*)	14.5 *	1.4
	Secreted protein acidic and cysteine rich	1.5 *	1

(*SPARC*)		
A disintegrin and metalloproteinase with thrombospondin motifs 1 (*ADAMTS1*)	2.8 *	1
Fibroblast growth factor receptor 2 (*FGFR2*)	Inf *	0.7
Mesothelin-like protein (*MSLN*)	9.2 *	9.7 *
Fibronectin (*FINC*)	1.9 *	1.4
Neural-cadherin (*N-CAD* or *CDH2*)	0.6 *	0.8
Neural cell adhesion molecule 1 (*NCAM1*)	0.2 *	0.8
Netrin-1 (*NTN1*)	1.7 *	1.4
Ephrin type-A receptor 4 (*EPHA4*)	2.1 *	0.8
N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase (*β3GNT1*)	2.5 *	1.3
Transcription factor AP-1 (*JUN*)	0.2 *	0.7
Partitioning defective 3 homolog (*PAR3*)	0 *	2.7 *
Biogenesis of lysosome-related organelles complex 1 subunit 1 (*BLOC1S1*)	1.1	1.5 *
Epidermal growth factor receptor substrate 15 (*EPS15L1*)	0.9	Inf *

Phosphatidylinositol-binding clathrin assembly protein (CALM)	2.2	33.3 *
Ras-related protein Rab-3A (*RAB3*)	1	1.5 *
Ras-related protein Rab-10 (*RAB10*)	1	1.4 *
Roundabout Homolog 2 (*ROBO2*)	0.9	1.5 *
T-lymphoma invasion and metastasis-inducing protein 1 (*TIAM1*)	1.2	1.6 *
Microtubule-associated protein 1A (*MAP1A*)	1.2	1.7 *
Microtubule-associated protein 1B (*MAP1B*)	1	1.7 *
Tripartite motif-containing protein 46 (*TRIM46*)	1	2.1 *

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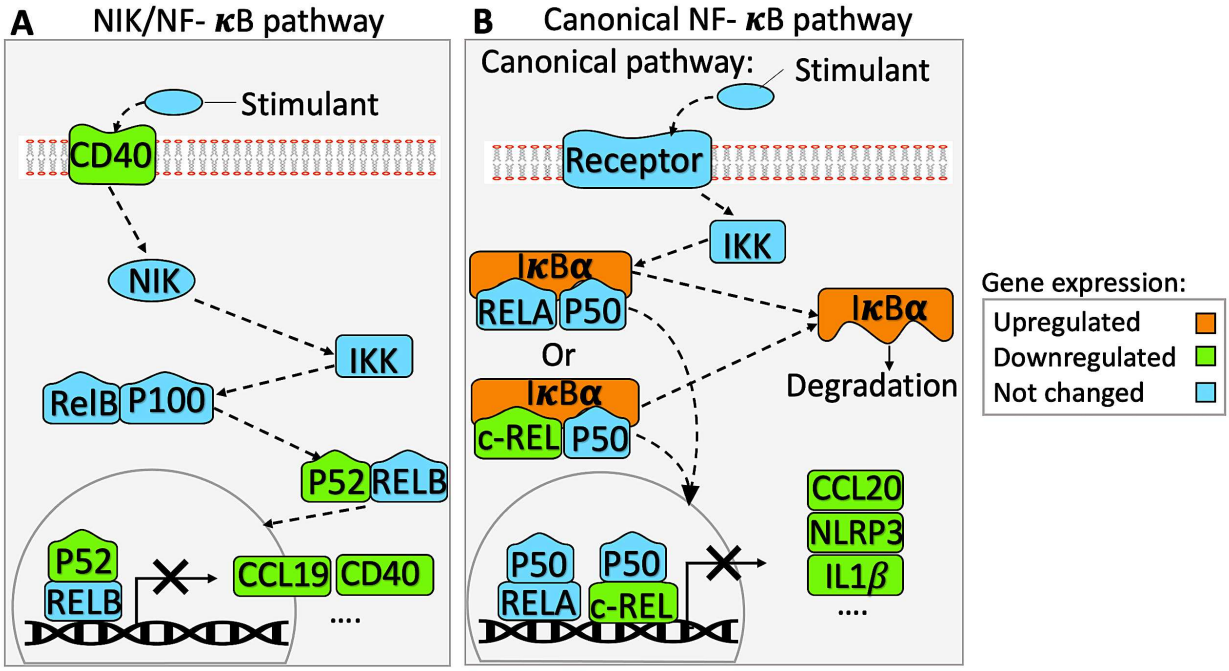

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809 Figure 3. Schematic representations of transcriptional alterations in NF-κB signaling pathways

810 following exposure to CuNPs in rainbow trout olfactory mucosa. (A) NIK/NF-κB signaling

811 pathway. (B) canonical NF-κB signaling pathway. The colour-coded legend represents the

812 transcription pattern of the differentially expressed genes.

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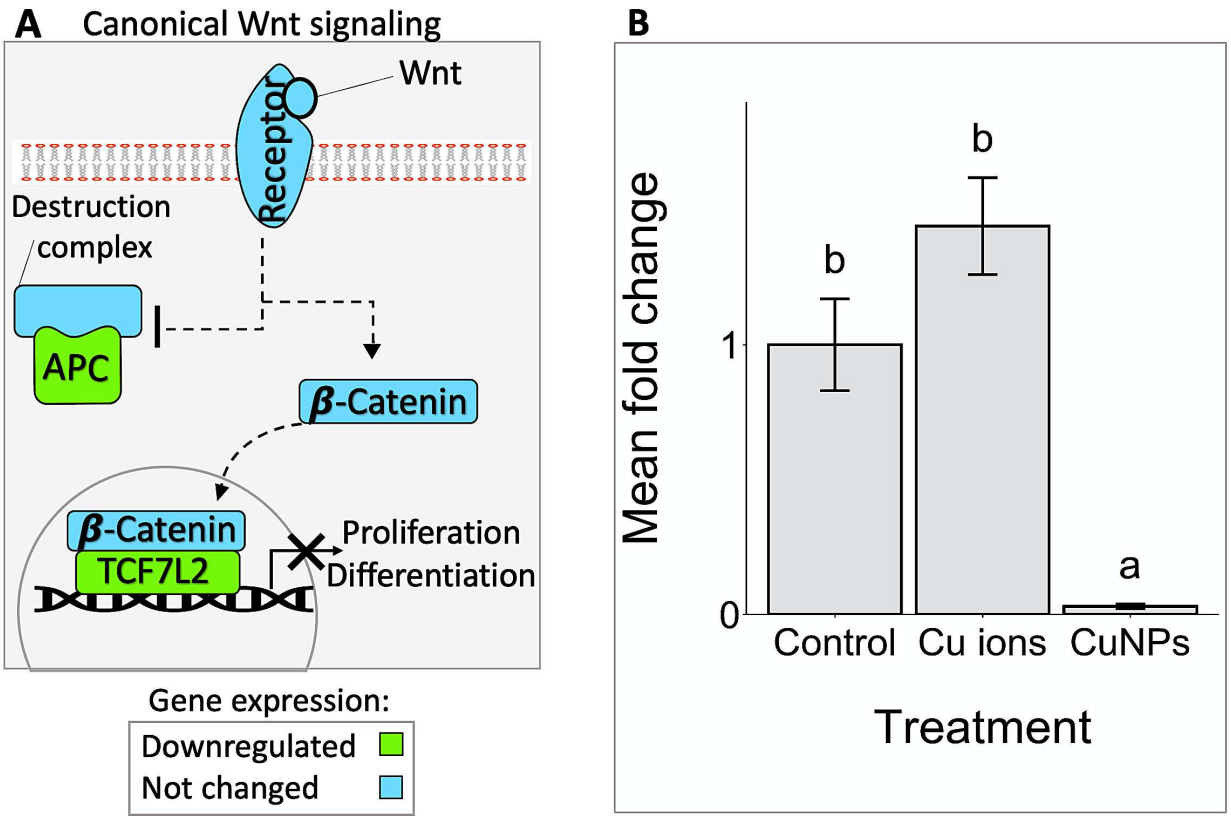

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816 Figure 4. Effect of CuNPs on the transcription of genes involved in canonical Wnt signaling

817 pathway in the rainbow trout olfactory mucosa. (A) Schematic representation of canonical Wnt

818 signaling pathway in the CuNP treatment. The colour-coded legend represents the transcription

819 pattern of the differentially expressed genes. (B) The relative expression of *TCF7L2* in response

820 to different Cu treatments in the olfactory mucosa. The gene expression was measured by qPCR.

821 Lower-case letters indicate significant differences ($p \leq 0.05$, error bars ± 1 SEM).

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823