Effects of urea on the olfactory reception in zebrafish (*Danio rerio*)

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Abstract

The effects of uremia on human olfactory functions have been clinically evaluated in various studies, even if to date it is not completely clarified which uremic toxins mediate these processes. Surprisingly, the role of the main molecule involved in uremia, urea indeed, has not been adequately investigated as other possible molecules may also be involved in uremic anosmia. The effects of urea on the olfaction have been evaluated in some clinical studies, but this is the first attempt to determine a direct action of urea on the olfactory epithelium of a vertebrate. *Danio rerio* adults were exposed to urea in different experiments to assess the effects on olfactory sensitivity and signal transduction. The analysis of the swimming speed has been used to evaluate the response to hypoxanthine 3-N-oxide (H3NO), a molecule that is known to elicit an olfactory-mediated alarm reaction in *D. rerio*. The presence and distribution of the G protein alpha subunit coupled to the olfactory receptors ($G\alpha_{olf}$) has been immunohistochemically investigated in the olfactory epithelium of control and urea-exposed *D. rerio*. Our findings showed that urea alters the response to H3NO of *D. rerio* with a quite rapid and reversible effect that appears to be independent from a mere interference of urea on the receptor-ligand binding. The $G\alpha_{olf}$ protein resulted increases after urea treatment, suggesting an effect of urea on its expression or degradation.

Introduction

The sense of smell is impaired in chronic renal disease.[1-4] However, which uremic toxin causes the olfactory impairment is unknown to date.[5] About urea, while studies correlated olfactory functions with urea blood levels,[1] others could not establish this correlation.[4] To our knowledge, in the literature[3,4] there are no reports about experimental approaches to urea effect on the olfaction of vertebrates.

Recently, the role of urea in olfaction was reported in two articles.[6,7] In these articles *Danio rerio* was used as a model to test the effects of urea on olfaction. Urea treatment in *D. rerio* affects the expression or degradation of the G protein alpha subunit olfactory type ($G\alpha_{olf}$) involved in olfactory transduction, causing an increased presence of this protein in the olfactory cilia.[6,7]

Bettini and co-authors studied the effects of urea on the *D. rerio* sensory mucosa through an immunohistochemical approach and described that urea, among other effects, appears to increase the $G\alpha_{olf}$-immunoreactivity even if it leads to sensory impairment directly affecting the olfactory organs. That appears to be in accordance with the functional olfactometric measurements previously reported in the literature.[6]

Aim of this study was to evaluate a general effect of urea on the sense of smell and its possible effect on the first steps of the olfaction process (signal reception and transduction).

In vertebrates, olfaction is mediated by different kinds of olfactory receptor neurons (ORNs) (*i.e.* ciliated, with microvilli and crypt neurons) and different G protein-coupled receptors families. The main olfactory organ in mammals hosts the ciliated ORNs, which bear receptor belonging to the olfactory receptor (ORs) family; this is because, in tetrapods, other kinds of ORNs and receptors are mostly located in another organ, the vomeronasal one.[8] In fish, generally, all the different ORNs and molecules are present in one organ, namely the olfactory organ.[9,10] In the present work we focus on the ciliated ORNs, ORs and their coupled $G\alpha_{olf}$. As these structures and molecules are present in all vertebrates, with the only possibly exception of chondrohychties,[11-13] the zebrafish *Danio rerio*, has been considered as a suitable model organism for the aim of this study.

The possible alteration of the olfactory sensitivity due to urea has been evaluated through the assessment of a behavioral endpoint. A behavioral endpoint can represent a rapid and effective way to detect changes at molecular level[14] and locomotory changes have been already used as behavioral endpoints in neurobiology of *D. rerio*.[15,16] The olfactory sensitivity and its possible alteration after urea treatment have been assessed on adult zebrafish using the alarm substance hypoxanthine 3-N-oxide (H3NO), a molecule able to elicit an olfaction-

mediated alarm reaction in some species of fish and also in zebrafish.[17,18] It has been indeed demonstrated that H3NO is detected by ciliated ORNs.[18]

The anxiogenic response to alarm substances can be used for very reliable endpoints since it is an innate reflex, and it has been already used as an endpoint of olfactory function.[14] The detection of H3NO has been evaluated developing – by means of a free software – a simple method of analysis of the swimming alteration due to the alarm reaction.

Also, the presence and/or functioning of ORs have been investigated after urea exposure. It is known that ORs are internalized after a specific ligand binding and this process can be highlighted using a marker molecule in the medium.[18,19] Exploiting this *accidental endocytosis*, the neutral red dye has been previously used by our group in order to highlight the presence and the odorant binding of ORs in control and urea-treated zebrafish larvae at 96 h after fertilization (hpf).[7] The stage of 96 hpf is characterized by a well developed olfactory epithelium[20] and the expression of ORs has been observed already at the embryonic stage 31 hpf.[21,22] We demonstrated that exposure to waterborne urea causes a higher urea content in the body fluids of developing zebrafish *D. rerio*[7] and it affects the presence of $G\alpha_{olf}$ in the olfactory cilia in both developing and adult *D. rerio*.[6,7] Moreover, chronic urea exposure of adult *D. rerio* caused alteration in epithelium thickness and in the number of receptor neurons, affecting differently the different neuron types.[6]

Thus, we here investigate the presence and distribution of $G\alpha_{olf}$ in fish exposed or not to urea, using immunohistochemical methods.

Materials and Methods

Fish treatment

D. rerio, males and females from a local supplier, were kept under controlled temperature, light and food parameters for one week. Four tanks of 10 L were prepared, two of them containing clean water (CTRL) and two containing urea 50 mM added to water. Fish was divided randomly in four groups of ten individuals, in the four tanks. The longest urea treatment lasted 5 d. One tank of CTRL and one tank of urea treatment were used for the olfactory assessment test (OAT) (Figure 1). The other two tanks, with the same conditions of the former two, were used as stocks of CTRL and urea-treated fish, in order to replace the sacrificed fish for the immunohistochemical analysis keeping the n=10 fish for the OAT until the end of the 5 d treatment.

Hypoxanthine 3-N-oxide

Hypoxanthine 3-N-oxide (cat. 19765-65-2; Synchem, Felsberg, Germany) was dissolved in phosphate buffered saline solution (PBS) in order to keep a constant 7.8 pH, which preserves the molecule from a rapid degradation, with a concentration of 20 μM (stock solution). The final concentration of H3NO in the fish tank for olfactory assessment was 5 nM.

Olfactory assessment test

The olfactory assessment tests (OATs) were performed in one tank of CTRL and one tank of urea treatment, each with 10 fish, using 5 nM H3NO in water. The differences between the swimming behaviors in normal condition and after H3NO administration were slightly appreciable in fish. Anyway, the presence of H3NO in the water led fishes to an overstated response to a further stimulus, like a visual one. Thus, the OATs consisted of two combined stimuli: the olfactory clue (H3NO) followed by a visual clue (waving hand) after 3 min from the H3NO administration.

The introduction of H3NO in the tanks has been designed in order to

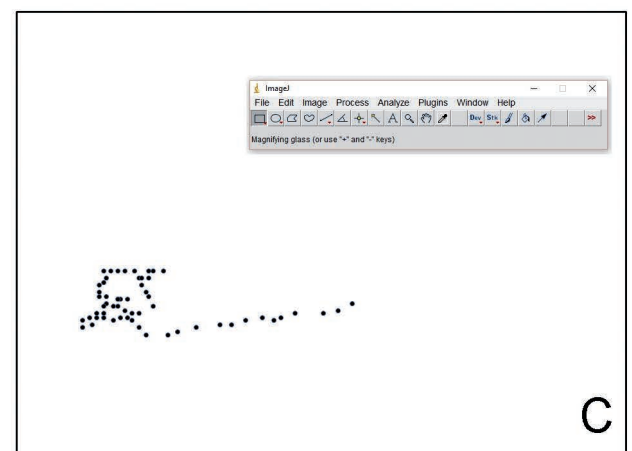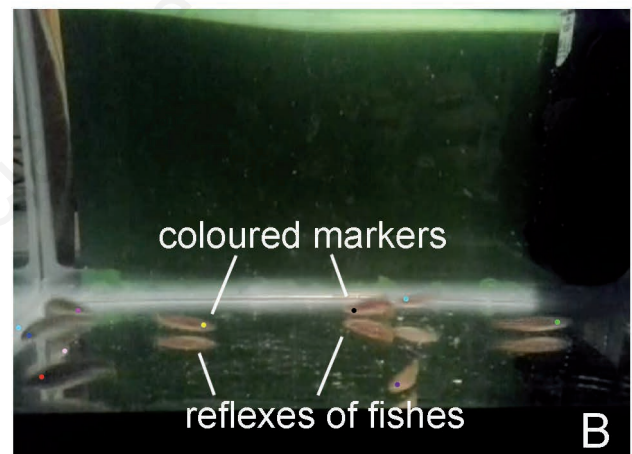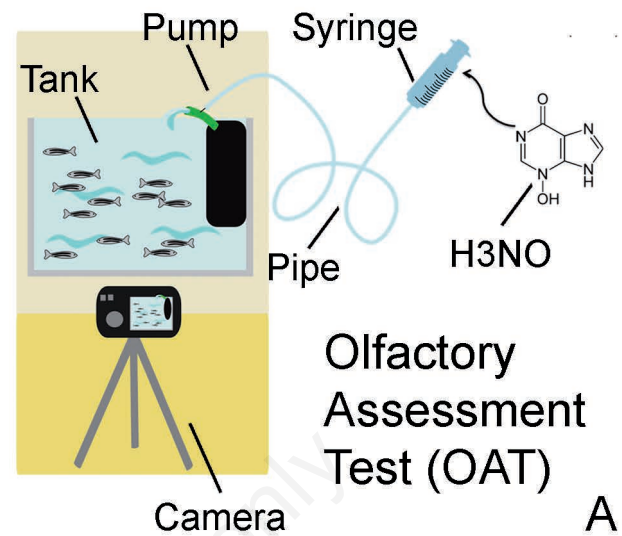**Olfactory Assessment Test (OAT)**

A

B

C

Figure 1. A) Schematization of the experimental device used to perform the olfactory assessment test; B) image of the tank with the coloured marker and the reflex of the fish that were manually filtered in each frame; C) detail of the partial overlapping of the series of positions of an individual fish that were analyzed with ImageJ software.

exclude any undesired stimulus other than the established chemical and visual ones. Tanks were without a lid and illuminated by lamps positioned above. The room was normally quite dark. In each tank, a needle was previously inserted in the rubber pipe that take a continuous water flow from the pump to the tank. Ten minutes before the experiment 2.5 mL of H3NO stock solution were drawn in a thin rubber pipe (2 m length) with a syringe and the pipe was applied to the needle. The camera was positioned in front of the tank. After 10 min, when fish were normally swimming, an operator at the side of the tank turned on the camera and started to pull the syringe causing a slow dripping of the H3NO solution in the water flow from the pump. Thus, fish could not perceive the dripping, or the different temperature of the H3NO solution and the chemical was quickly diffused in the tank. After 3 min from the H3NO administration, the operator previously hidden at the side of the tank, waved the hand above the tank with three rapid movements. The whole operation was recorded by the camera and lasted 2 min after the H3NO injection.

The OATs were performed at different times of the experiments, and 2/3 of the water (or the water with 50 mM urea) was exchanged each time in order to quickly remove most H3NO. The OAT was performed on fish at different experimental conditions: CTRL, fish exposed to urea 50 mM for 5 min (U5m), fish exposed to urea 50 mM for 20 min (U20 m), fish exposed to urea 50 mM for 5 d (U5d), fish exposed to urea 50 mM for 5 d and then placed to recover in clean water for 15 min (U5dR15m), fish exposed to urea 50 mM for 5 d and then placed to recover in clean water for 1 h (U5dR1h) (Figure 1).

Evaluation of swimming alteration

Only one camera was used, thus only the movements of fish on two dimensions (x: length of the tank; y: depth of the water) were considered. No information about the movements in the other axis (z: width of the tank) was taken into account. The first 12 sec, after the visual stimulus following the H3NO injection, were the most significant for the detection of swimming alterations. Thus, for each OAT, quantitative analyses were performed in that time lapse. The recorded video was managed using the free software Avidemux2 (http://fixounet.free.fr/avidemux/) and the first 12 sec after the stimuli (360 frames) were used to create an image stack using the open source software ImageJ (http://rsb.info.nih.gov/ij/). For each frame in the stack, each of the ten fish was labeled with a colored dot (one different color for each fish). Using the *Color Threshold* and *Analyze Particles* tools we obtained the coordinates of each fish in each frame. The coordinates of each fish in the 360 frames were used to calculate the speed every 10 frames, thus 36 speed records every 12-sec video have been considered. As blanks, 12 sec of swimming after the visual stimulus but without H3NO have been considered in control and U5d fish. A graphic representation of the fish speeds in the different experiments and the calculation of the standard deviation for the speed values have been made using Microsoft Office Excel (Microsoft, Redmond, WA, USA).

Immunohistochemistry

After the OATs of CTRL, U20m, U5d, U5d1h (representing the control, the longest time of treatment and the experimental conditions that caused a variation in the sensitivity to H3NO compared to the precedent condition) three adult fish were deeply anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate salt (Sigma Aldrich, St. Louis, MO, USA), then rapidly decapitated, and the head were fixed in paraformaldehyde at 4°C for 24 h, thoroughly washed in phosphate buffer saline (PBS) 0.1 M pH 7.4, decalcified for 24 h on Osteodec (Bio-Optica, Milan, Italy), dehydrated in ethanol, paraffin embedded and 5 μm sectioned. Then, the three sacrificed fishes were replaced with other three fishes from the stock tanks of control and of urea treatment.

Histological sections from adult fish (CTRL, U20m, U5d, U5d1h) were used to immunodetect of $G\alpha_{olf}$. A rabbit anti-$G\alpha$s/olf primary antibody (sc-383; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used (1:200 in PBS). This antibody has been widely used in non-mammalian vertebrates and also invertebrates[9,10,23,24] because G protein alpha subunits are conserved in evolution.

As secondary antibody, an AlexaFluor488-conjugated anti-rabbit antiserum (Invitrogen, Carlsbad, CA, USA) was used (1:800 in PBS). Negative controls were always performed by omitting the primary antibody and by neutralizing the primary antibody with its antigens, the blocking peptides purchased by the same company (sc-383P; Santa Cruz Biotechnology).

In silico evaluation of the antibody on zebrafish protein

This evaluation was previously performed by Ferrando and co-authors.[7] For reader utility we report the evaluation method used. Santa Cruz Biotechnology does not make the exact sequence of antigen for the anti-$G\alpha_{s/olf}$ antibody (sc-383) available, but shares a 50-aa range of a mammalian protein, within which the antigen is localized. The epitope of the anti-$G\alpha_{s/olf}$ is about 15-25 aa long and mapped within the last 50 C-terminal aa of the rat protein with Uniprot accession number P38406. The online software ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for the alignment of the C terminus of P38406 and the zebrafish $G\alpha_{olf}$ (Q5U3H6).

Results

Olfactory assessment test

No fish died during the 5 days of urea treatment, nor in the CTRL. The results of blanks for the OATs, that is the response of CTRL and U5d fish just to the visual stimulus, are reported in Figure 2. Fish of CTRL, after H3NO and visual stimuli, showed a sudden burst of swimming, testifying the olfactory reception of H3NO (Figure 2A). The U5m fish showed a reaction to the stimuli similar to the CTRL (Figure 2B). After 15 min, the U20m did not show an appreciable response to the stimuli (Figure 2C). The unresponsiveness lasted during the 5 d of treatment, as reported for U5d (Figure 2D). Fifteen minutes of recovery in clean water did not return the responsiveness to H3NO (Figure 2E), while one hour is enough to show an appreciable response to the stimuli, still not equal to the control (Figure 2F). Our experiments showed that the average of swimming speed does not change significantly in normal swimming or during alarm behavior, because fishes alternated very rapid and very slow movements. The standard deviations of the speed measurements (360 speed measurement=36 for each of the 10 fishes during the 12 seconds of video) seemed more explanatory and are reported, for each experiment, in Table 1.

In addition to the variation of swimming speed, two further behavioral endpoints were considered in order to evaluate the olfactory sensitivity to H3NO: the variation of clustering and of frequency of direction change. As no statistically significant results were obtained, those data are not reported in the present work.

G protein alpha subunit olfactory type-immunoreactivity

Immunofluorescence for $G\alpha_{olf}$ has been highlighted in the olfactory cilia of all fish. Although immunohistochemistry is not a quantitative technique, it is possible to note that the immunoreactivity was similar in CTRL and U20m fish (Figure 3A and B). It was more intense in U5d and U5dR1h fish (Figure 3C and D). Both the omitting and the preabsorption of the primary antibody prevented the immunostaining.
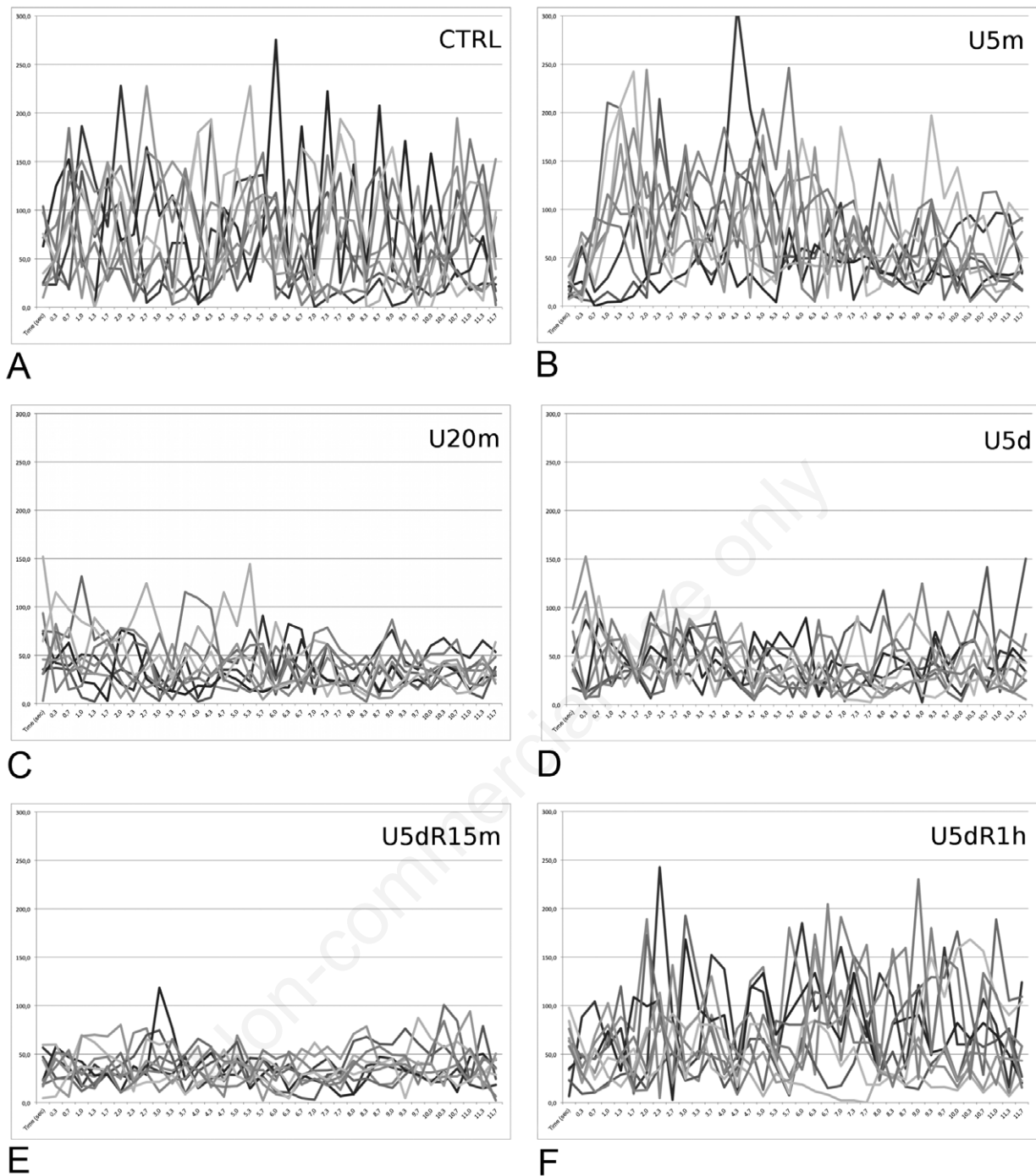
Figure 2. Diagrams of the speed of ten adult zebrafish, calculated every 10 frames of a 12-sec video, immediately after chemical (H3NO)+visual stimuli. Each line represents a fish. Time (s) on X axis. Speed (mm/s) on Y axis. A) control (CTRL); B) urea 50 mM for 5 min (U5m); C) urea 50 mM for 20 min (U20m); D) urea 50 mM for 5 d (U5d); E) recovery in clean water for 15 min after urea 50 mM for 5 d (U5dR15m); F) recovery in clean water for 1 h after urea 50 mM for 5 d (U5dR1h).

Table 1. Standard deviations of the speed measurements, calculated as mm/s, 36 times in 12 seconds (every 0.3 seconds) for ten fish.

	CTRL blank	U5d blank	CTRL OAT	U5m OAT	U20m OAT	U5d OAT	U5dR15m OAT	U5dr1h OAT
SD	27.7	19.4	53.4	51.3	24.9	26.7	18.1	46.3

SD, standard deviation; CTRL blank, visual stimulus of control fish; U5d blank, visual stimulus on fish exposed to urea 50 mM for 5 d; CTRL OAT, chemical+visual stimuli on control fish; U5m OAT, chemical+visual stimuli on fish exposed to urea for 5 min; U20m OAT, chemical+visual stimuli on fish exposed to urea for 20 min; U5d OAT, chemical+visual stimuli on fish exposed to urea for 5 d; U5dR15m OAT, chemical+visual stimuli on fish exposed to urea for 5 d and let recover in clean water for 15 min; U5dR1h OAT, chemical+visual stimuli on fish exposed to urea for 5 d and let recover in clean water for 1 h.

Proteins alignment

The last 50 C-terminus aa of the rat protein identified by the Uniprot accession number P38406 (Gα$_{olf}$) has been aligned, using the online software ClustalW2, with the C terminus of the zebrafish Gα$_{olf}$ (Q5U3H6) (Figure 4) showing a high identity correspondence.

Discussion

From our data we can suggest that the visual stimulus we used in these experiments is not able, alone, to trigger an alarm response detectable by
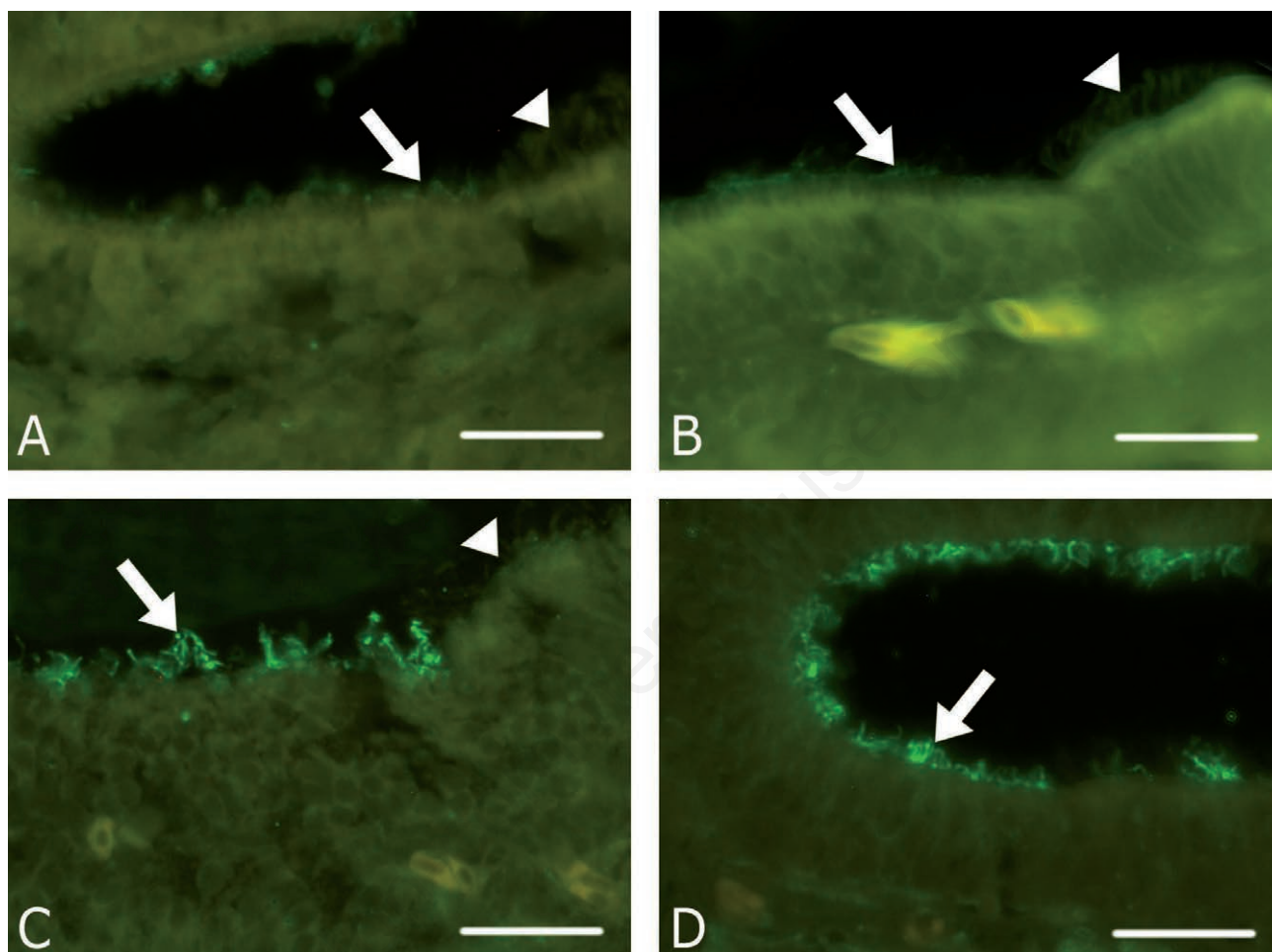

Figure 3. Immunofluorescence for Gα$_{olf}$ in the olfactory organ of zebrafish. A) Control fish. The immunoreactivity (ir) is present in the olfactory cilia (arrow) and absent from non-sensory cilia (arrowhead). B) Fish exposed to urea for 20 min. The ir is very similar to the control. C) Fish exposed to urea for 5 d. The ir in the olfactory cilia is more intense than in the control. D) Fish exposed to urea for 5 d and then recovered in clean water for 1 h. The ir in the olfactory cilia is very similar to the 5-d urea treated. Scale bars=20 μm.

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sp|P38406|GNAL_RAT       EYANYTVPEDATPDAGEDPKVTRAKFFIRDLFLRISTATGDGKHYCYPHF 350
tr|Q5U3H6|Q5U3H6_DANRE   EYARYTLPPEATPDPGEDPKVSRAKFFIRDEFLKISTASGTDKHYCYPHF 348
                         ***.**:*  :****.******:*******  **:****:*  .********

sp|P38406|GNAL_RAT       TCAVDTENIRRVFNDCRDIIQRMHLKQYELL 381
tr|Q5U3H6|Q5U3H6_DANRE   TCAVDTENIRHVFNDCRDIIQRMHLRQYELL 379
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Figure 4. Clustalw2 alignment of the peptide used to raise the commercial antiserum against Gα$_{olf}$ with the sequence of correspondent zebrafish proteins. The alignment of the last 50 aa of rat Gα$_{olf}$ protein (accession P38406) with zebrafish Gα$_{olf}$ (accession Q5U3H6) shows high similarity of the sequences.

the swimming analysis we performed. Thus, the alarm response we recorded in fish after the visual stimulus is due to the anxiogenic effect of H3NO, which is known to explicate through the ciliated ORNs.

We can exclude that urea in the water could somehow react with H3NO, rendering it biologically unavailable, because the fish treated with urea for 5 min were alarmed by the H3NO.

It must be considered that the impaired alarm response to H3NO after urea exposure could be due to urea effects at different levels, for example neurological more than sensorial. The use of H3NO that normally trigger an innate reflex, should avoid at least the detection of memory and learning impairment.[14]

The recorded effect of urea on the H3NO -triggered alarm response is not instantaneous as after 5 min it is still undetectable, but after 20 min is complete, as we are not able to recognize any alarm reaction in fish.

The great majority of the evaluations of the olfactory sensitivity after the exposure to a chemical were performed after a time of exposition of some hours or days,[25-28] but also exposures of tens of minutes have been evaluated, showing that some chemicals, as for example copper, are effective on a quite short time.[29,30]

After 5 days of urea treatment fish have no response to H3NO, as observed after just 20 min, suggesting that, whatever the mechanism of urea interference with the alarm response, it is not susceptible to adaptation and recovery during the treatment. Back to clean water, fish are able to recover an appreciable response to H3NO in one hour.

Even if the immunohistochemistry is not a quantitative technique, a more intense immunoreactivity for $G\alpha_{olf}$ has been detected after some days of urea exposure, in the olfactory cilia *D. rerio*. Our observation showed that one hour of recovery, which restores the capability of an alarm response to H3NO, did not make the immunoreactitvity back to the intensity of the control. The major presence of $G\alpha_{olf}$ could be due to an increased synthesis or to a decreased degradation. In fact, in mammalian brain a lower degradation of $G\alpha_{olf}$ has been recognized as the cause of its increased presence.[31]

Conclusions

We can conclude that urea treatment has a direct effect (after just 20 min with a recovery of just 1 h) on the alarm response to H3NO, and possibly on olfactory sensitivity. This effect is not due to the effect of ORs presence and functioning. Moreover, urea exposure increased the presence of $G\alpha_{olf}$. We observed a reduced olfactory sensitivity after 20 min of urea treatment when the level of $G\alpha_{olf}$ seemed the same as that of CTRL. We also observed a sensitivity recovery in U5dR1h when the level of $G\alpha_{olf}$ seemed higher than the control. Thus, we can state that the olfactory sensitivity is not directly dependent by the observed $G\alpha_{olf}$ immunoreactivity. The $G\alpha_{olf}$ variations detected may be explained as an attempt to restore the equilibrium following the urea effect.[14]

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