Effect of hippocampal 6-OHDA lesions on the contextual modulation of taste recognition memory.

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Abstract

Taste recognition memory is evident in rodents because the initial neophobia to novel tastes attenuates across exposures as the taste becomes familiar and safe. This attenuation of taste neophobia (AN) is context-dependent and an auditory background change could induce the recovery of the neophobic response. The AN auditory context-dependency requires the hippocampal integrity but the neurochemical mechanisms underlying the interaction with the taste memory circuit remain unexplored. We have applied pharmacological intervention by 6-hidroxydopamine (6-OHDA) hippocampal lesion for assessing the role of catecholamines in the hippocampal system to Wistar rats that drank a novel 3% vinegar solution for several consecutive days. Additionally, we manipulated the auditory background as a context that could either change or remain constant across all the drinking sessions. We found that a disruption of the context-dependent AN was induced by intracerebral administration of 6-OHDA targeted to the ventral CA1 hippocampus (vCA1). We conclude that the ability of the auditory context to modulate taste recognition memory involves the catecholaminergic activity in the ventral hippocampal circuit for the proper acquisition of safe taste memory.

Taste recognition memory is a robust paradigm for studying the neural mechanisms of learning and memory processes regulating intake in rodents [1]. Familiar tastes are recognized as either aversive or safe

depending on the consequences of previous encounters. Safe taste recognition memory is evident as the initial neophobia to novel tastes is attenuated upon repeated exposures, leading to increased consumption. The attenuation of taste neophobia (AN) has proven to be context-dependent since it is disrupted by a spatial context change in rats [2]. Moreover, we have previously reported that the non-spatial auditory context-dependency of AN in mice depends on the hippocampal CA1 field integrity [3].

The role of the hippocampus in context processing has been related to its relevance for the formation of multiple complex representations of stimuli [4]. Although the hippocampal role in the contextual information of taste learning has been thoroughly demonstrated in taste aversion learning [5,6] the relevant circuits mediating its interaction with the brain areas involved in safe taste learning remain unexplored. Taste neophobia and AN depend on a brain circuit that includes the insular cortex [7,8], basolateral amygdala [9], piriform cortex [10], perirhinal cortex [11] and nucleus accumbens [12,13].

The relevant interaction between memory and taste processing circuits might be mediated by the dopaminergic activity. Dopamine (DA) plays a critical role in the contextual memory formation [14] and taste learning formation [15]. Also, DA has been linked to AN [12] and other types of taste learning [16]. The hippocampal-ventral tegmental area projections, mediated by DA, have been proposed as a novelty-triggered memory consolidation mechanism [17]. The potential role of dopaminergic hippocampal innervations in AN is in accordance with the disruption of the contextual modulation of AN after the systemic administration of D1 dopamine receptors antagonists in mice [18]. Moreover, the ventral region of the hippocampus is highly innervated by catecholamine terminals coming from either the mesocorticolimbic pathway (nucleus accumbens and ventral tegmental area) or the locus coeruleus [4,14,19],

In the present experiment, we explore the role of the hippocampal catecholaminergic activity in the auditory context-dependency of AN in rats through the depletion of catecholamine terminals of the ventral CA1 subfield of the hippocampus by i.c. administration of 6-hydroxydopamine (6-OHDA). We hypothesize that hippocampal catecholaminergic innervations are required for the context modulation of AN and its depletion should disrupt the contextual dependency of AN.

A total number of 40 adult male Wistar rats (Charles River, France) were used in this series of experiments. All animals were housed individually and maintained on a 12-hour dark-light cycle (lights from 8:00 am to 8:00 pm) during the behavioral procedure. All procedures were approved by the University of

Granada Ethics Committee for Animal Research and Junta de Andalucía (CEEA17-02-15-195) and were in accordance with the European Communities Council Directive 86/609/EEC.

Ventral hippocampus and sham catecholamine depletion were randomly assigned to experimental groups. Thus, four groups were used to assess the impact of lesion: *Sham-Vinegar-Same Sound* (*n*=8), *Sham-Vinegar-Different Sound* (*n*=8), *6OHDA-Vinegar-Same Sound* (*n*=8) and *6OHDA-Vinegar-Different Sound* (*n*=8). For this purpose, all animals were i.c. injected with either 6-OHDA or vehicle (sterile 0.9% NaCl solution) into the ventral CA1 region of the hippocampus one week before the behavioral procedure started. Surgery was performed under general anesthesia with a mixture of ketamine and medetomidine (0.1% b.w.). The animals were randomly assigned to one of two groups: 6-OHDA and sham. They were placed in a stereotaxic apparatus (Stoeling Co. Instrument, Word Dale, IL, USA) with bregma and lambda at the same height. Small trephine openings were drilled in the exposed skull to perform two bilateral injections of either 6-OHDA (12 µg/µl dissolved in phosphate-buffered saline (PBS) plus 0.01% L-ascorbic acid) or sterile phosphate-buffered solution (PBS; pH= 7.4) through 30 gauge injection needles that were connected to 10µl Hamilton syringes so that 0.5µl was infused in each hemisphere at a rate of 0.5µl/min using an injection pump (Harvard Apparatus, Holliston, MA, USA). The needles were left in place for an additional 90 seconds before being slowly withdrawn. The stereotaxic coordinates targeted dorsal CA1 according to Paxinos and Watson´s rat brain atlas (2007) relative to bregma (AP: -4.8; ML: ±5; DV:-7.6 mm for the first injection and AP: -5.3; ML: ±5.4 and DV: -8 mm for the second injection). The skin was sutured and covered with povidone. After the surgery, all animals received additional s.c. injections of 5% Baytril and Bupac (0.1ml) for four consecutive days to reduce post-surgical pain and prevent infection.

After a period of 8-12 days, all the animals received the same behavioral procedure, a three-phase protocol: Baseline (5 days), Phase I (1 day), and Phase II (2-4 days) (**Figure 1A**). During all the procedure the animals had a 15-minute daily drinking sessions where liquid was available and the amount of ingested was recorded. An experimentally-controlled auditory background was continuously present during all the drinking sessions (including Baseline, Phase I, and Phase II). Two different sounds were used and counterbalanced amongst the subjects. One sound was a pure 600 Hz tone (PT) consisting of 3-second pulses with an interstimulus interval (ISI) of 3 seconds (70 dB). The second sound was Gaussian white noise (WN) consisting of 2-second pulses with an ISI of 4 seconds (70 dB). Both sounds were created using MATLAB.

Each day two speakers were positioned approximately at one meter from the rack containing the homecages. Immediately after the sound started the tubes containing the solution were placed. After 15 minutes, the tubes were removed and the sound stopped. All animals received a rehydration session with free water access where sounds were not present.

During Phases I and II all rats had access to the 3% cider vinegar solution during the 15-minute drinking sessions. The rats assigned to the *Same Sound* groups were only exposed to one of the two auditory cues (either the PT or the WN). The rats assigned to the *Different Sound* groups experienced a change in the auditory background in Phase II. Thus, the auditory cue present during Phase II was different from that used on Baseline and Phase I (vinegar day 1). Due to counterbalancing half of the animals changed from PT to WN and the other half changed from WN to PT.

Additionally, in order to look for possible confounding impact of the auditory background change over the drinking behavior, two groups of rats (n=8 each) were exposed to the auditory backgrounds similarly to the previous subjects, while they were allowed to drink water.

No differences between Sham and 6-OHDA groups were observed during the water Baseline (see **statistical analyses** and **Supplemental Figure 1**), which allows to discard nonspecific effects of the lesion on drinking behavior. All groups consumed lower amounts of water on Day 1 compared to Days 2, 3, and 4 (p<.001) during the Baseline which is an indicative of a similar adaptation to the water deprivation procedure in both groups.

All groups showed neophobia to the cider vinegar solution represented by an abrupt drop in consumption on the first day of presentation and a progressive intake increase over the following days. Sham groups exhibit the context-dependency of AN in the second exposure to vinegar when the novel auditory background became familiar with no differences between Same and Different groups. However, the ventral hippocampal catecholamine depletion interfered with this effect as vinegar intake increased on Day 2 despite the context change (**Figure 1B**). A one-way ANOVA comparing vinegar intake on Day 2 reveiled a significant effect of group [$F(3,28)=8.275$; $p=.004$]. Post-hoc analyses by Tukey´s multiple comparisons tests confirmed lower vinegar intake of the Sham-Different group than the rest of the groups (all p´s <.05) and no other difference was observed between the rest of groups. This result is similar to that previously reported after hippocampal CA1 NMDA lesions [3].

Moreover, in order to discard any potential unspecific stress/arousal effect of the sound change on drinking behavior a 4 X 2 (Day X Context Change) repeated measures ANOVAs was performed in two groups of rats that were exposed only to water and were exposed to the sound change on Day 4 (**Figure 1C**). The analysis did not reveal any significant effect or interaction (all p´s>.05), showing that all the groups drank similar amounts of water regardless the change in the auditory background.

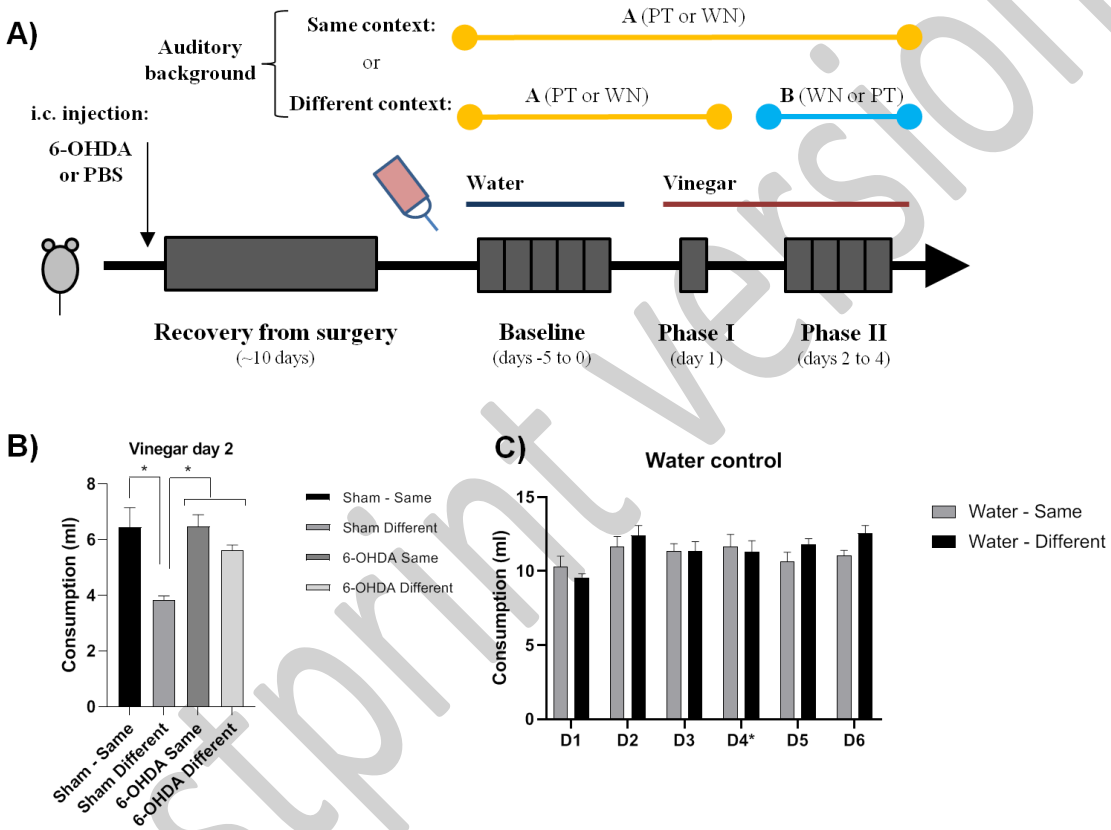

Figure 1. A) Schematic representation of the behavioral protocol used in the present experiment. **B)** shows the mean consumption (±SEM) of vinegar during Day 2. **C)** No effect of the context change on the water intake of an additional group of rats. The auditory backgrounds used to define contexts were counterbalanced. The symbol * represents statistically significant differences (p<.05) compared to Vinegar 1 within the same injection group.

All the animals were euthanized ~24 hours after the last drinking session. They were deeply anesthetized with sodium pentobarbital (100mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were removed and placed in a 4% paraformaldehyde solution for 4 h at 4ºC before being transferred to a 30% sucrose solution until they sank for cryoprotection. Coronal sections were cut at 20µm in a cryostat (Leica CM1900). Tissue sections were then rinsed in phosphate-buffered saline

(PBS 0.01 M, pH 7.4) and incubated in a solution of 3% normal goat serum and 0.5% PBS twen20 for 1 hour. Slices were transferred to a rabbit anti-tyrosine hydroxylase primary antibody (1:1000, #AB152, MERCK USA) overnight at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (Goat Anti-Rabbit IgG (H+L) cross-adsorbed Secondary Antibody, Alexa fluor 488, 1:500, #A-11008) for 120 minutes at room temperature. Primary and secondary antibody solutions were mixed in a solution of 2% normal goat serum, and 0.5% PBS tween20. Finally they were rinsed, mounted on gelatine-bubbed slides, and cover-slipped using DAPI mounting medium.

The analysis of 6-OHDA induced lesion was assessed using a Fluorescence module on a Microscope (Olympus BX41). Slices containing the ventral CA1 region were identified using the *NeuroLucida* Software (mbf Bioscience) from coronal sections located at the level of approximately at -5 mm relative to Bregma according to Paxinos and Watson (2007). Within each section microphotographs at 20X magnification were captured using the blue (DAPI) and green (TH) filters for the ventral CA1 hippocampal region that covered the nuclei (see **Figure 2A,B**). Tyrosine hydroxylase (TH+) terminals quantification confirmed that the 6-OHDA administration resulted in a selective decreased of TH+ fibers number within the hippocampus (both in the dorsal and ventral CA1 regions), but not in control areas such as the ventral tegmental area (VTA) compared with sham-injected animals. Moreover, the analyses revealed no differences in the total number of DAPI stained cell nuclei present in the brain samples, indicating that the injection of 6-OHDA selectively affected catecholamine terminals rather than a global and unspecific lesion of the area (**Figure 2C**). Other hippocampal subfields such as CA3 and dentate gyrus exhibited catecholamine depletion (**Figure 2D**).

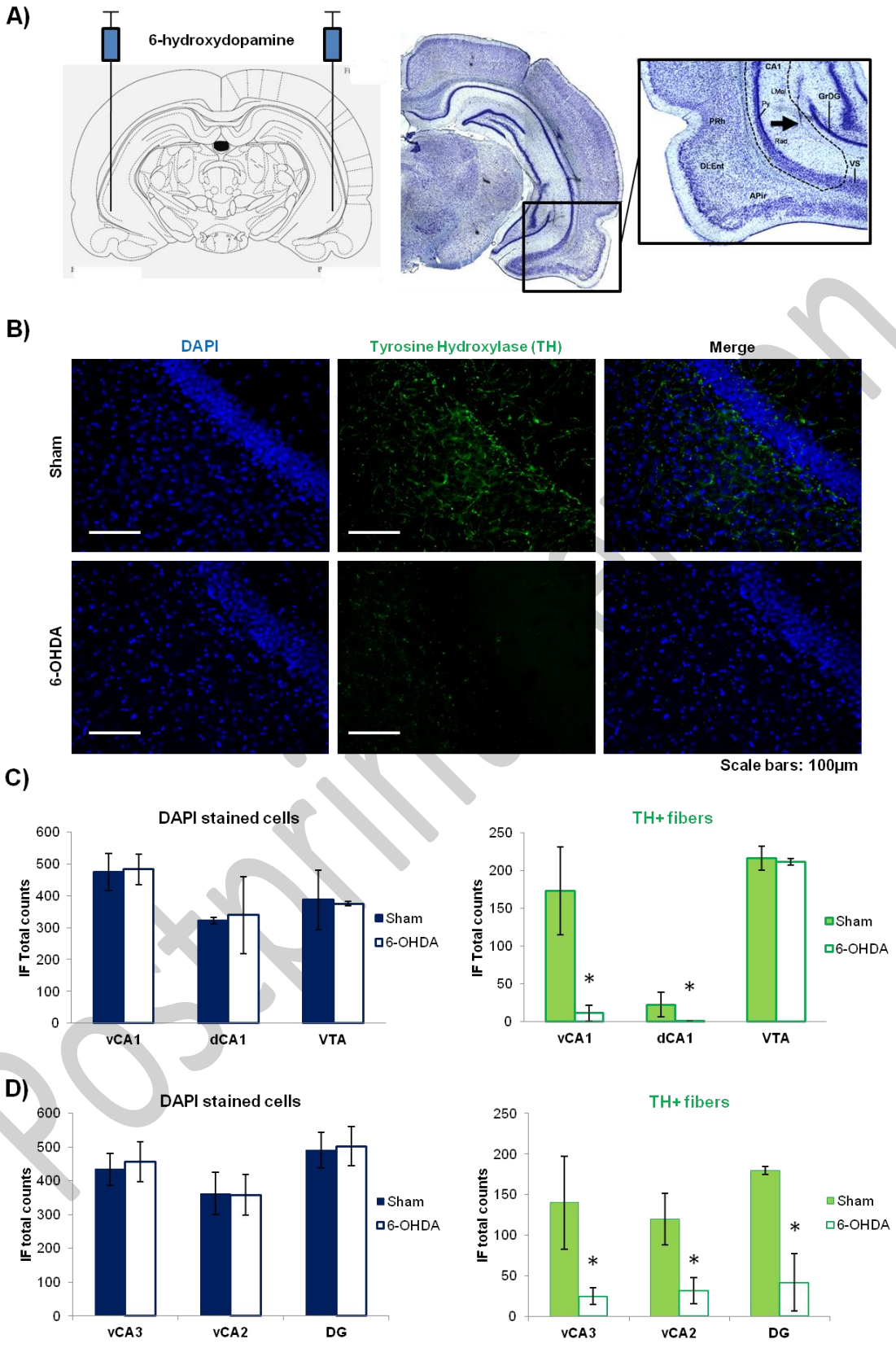

Figure 2. (A) Representation of the injection site of 6-OHDA (black arrow shows the tip of the microinjector). **B)** Representative microphotographs of immunofluorescence of Tyrosine Hydroxylase (TH+), green color, counterstained with DAPI of the ventral CA1 region in a Sham and 6-OHDA animals. **C)** Mean ±SEM of the total counts of DAPI stained cells (left) and TH+ stained fibers (right)

of different brain regions: dorsal and ventral CA1 region of the hippocampus and ventral tegmental area (VTA) in the Sham and 6-OHDA groups. D) Mean ±SEM of the total counts (n=3 per group) of DAPI stained cells (left) and TH+ stained fibers (right) indicating depletion spread from CA1 to CA3, CA2 and dentate gyrus (DG). The symbol * represents statistically significant differences ($p<.05$) compared to the Sham group.

The present data indicate that auditory contextual changes could modulate the AN behavioral expression and that catecholamine activity within the ventral hippocampus might be critical for it. This is consistent with the selective role of the catecholaminergic activity in the hippocampus but not the insular cortex for processing contextual information in other recognition memory tasks [26]. The fact that the auditory background provides contextual information influencing AN adds support to our previous reports [3,18]. The modulation of AN by the auditory context was assessed using two different auditory backgrounds. Changing the auditory background in the second taste solution exposure disrupted AN while the groups under a constant auditory background exhibited complete AN on day 2. Given that the groups subjected to the context change exhibited AN on day 3 in which the second context was already familiar, it can be conceived that the memories of both context and taste have been associated to form a context-dependent safe taste memory, whatever the learning mechanisms involved. This is in accordance with previous results demonstrating the spatial context-dependency of AN [2] and it extends the phenomenon to non-spatial auditory contextual cues. Hence, this is consistent with a wide definition of context that includes the auditory modality in addition to the cues previously used such as visual [2] and temporal information [23].

Therefore, although we cannot discard a role of norepinephrine or epinephrine, it could be hypothesized that the AN disruption induced by changing the auditory background might be due to the effect of the context change on the hippocampal dopaminergic activity given our previous reports relating dopamine with AN [24] as well as with its auditory context dependence [21]. In fact, DA has been reported to be crucial for consolidating contextual memories in the hippocampus [14,21,22]. We showed that catecholaminergic depletion of the ventral hippocampus CA1 region, using 6-OHDA, induce the absence of the AN context-dependency since the familiar taste consumption increased on day 2 despite the auditory context change. Given the fact that the catecholaminergic depletion extended to various ventral hippocampal areas such as CA3, CA2 and DG with different potential roles in acquisition, consolidation, storage and retrieval of memory [4] a definitive conclusion on the process responsible of the impairment found cannot be achieved. Several

functions have been proposed for CA1, CA3 and DG, such as novelty detection, temporal patterns completion/separation, context associations, working and episodic memories [27, 28] that could explain the results, Thus, it seems that in those 6-OHDA animals was impaired the association between taste and auditory cues whatever the specific subfield contribution and the nature of the process impaired. This is consistent with the widely accepted hippocampal role in learning and memory processes depending on contextual cues [23]. However, we cannot discard that 6-OHDA lesioned rats associated taste and the auditory cue but they were unable to retainor retrieve this information and use it to guide behavior. Accordingly, the depletion of dopaminergic hippocampal projections to the prefrontal cortex could be responsible of behavioral inhibition and cognitive control deficits as we have reported the involvement of the medial prefrontal cortex in AN [26]. Little is known about the anatomical pathways that mediate the interaction between the hippocampus and the anatomical circuit responsible for the safe memory taste formation. Although more research is needed to understand how catecholamines in the hippocampus interact with the neural circuits involved in regulating consummatory behavior, these results contribute to identifying the brain network that underlies the formation of complex taste memories modulated by the environmental cues that go beyond spatial information processing.

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Statistical analyses

1 Baseline: Water consumption.

A global Mixed 4 x 2 x 2 x 2 (*Day* X *Injection* X *Context Change*) repeated measures ANOVA comparing the amount of water intake between all the groups during the four days of baseline (BL) revealed only a significant effect of *Day* [$F(3,84)=33.36$; $p<.001$]. No other effect or interaction was significant (all *p's*>.5). Further analyses of the main effect *Day* using Bonferroni-corrected tests revealed that all groups consumed less amounts of water on BL Day 1 compared to BL Days 2, 3 and 4 ($p<.001$).

2 Vinegar consumption: Auditory context change and 6-OHDA lesions.

A global Mixed 4 X 2 X 2 (*Day* X *Injection* X *Context Change*) repeated measures ANOVA that compared the intake of vinegar amongst the groups on the four days after the baseline period was applied.

There was a significant effect of the main factor *Day* [F(3,72)=126.72; p<.001], the interactions *Day* X *Context Change* [F(3,72)=7.69; p<.001] and *Day* X *Injection* X *Context Change* [F(3,72)=3.589; p=.018].

To analyze the interactions additional 4 X 2 (*Day* X *Context Change*) repeated measures ANOVAs were performed for the *Sham* and *6OHDA* groups separately. The analysis for the *Sham* groups confirmed a significant effect of the main factors *Day* [F(3,36)=51.87; p<.001] as well as the *Day* X *Context Change* interaction [F(3,36)=8.25; p<.001]. Analysis of the interaction by additional repeated measures ANOVAs of the vinegar consumption was performed on the factor *Day* for each of the *Context Change* groups separately. The analyses confirmed a significant effect of *Day* in the *Sham-Vinegar-Same Context* group [F(3,18)=25.73; p<.001] as well as the *Sham-Vinegar-Different Context* [F(3,18)=35.18; p<.001], thus indicating AN. Further comparisons using Bonferroni-corrected tests identified significantly less vinegar was consumed on Day 1 compared to Days 2, 3 and 4 (all *p's*<.01) in the *Sham-Vinegar-Same Context* group, and this confirms that the neophobic response to the vinegar taste was completely attenuated on Day 2 and its consumption remained stable across the rest of days. In contrast, the same analysis performed in the *Sham-Vinegar-Different Context* group identified that the amount of vinegar consumed on Days 1 and 2 were indistinguishable (p=1) and less than on Days 3 and 4 (*p's*≤.007). Thus unlike the rats that did not experience a change of auditory background, the animals that experienced the change maintained the neophobic response for one more day; the attenuation of taste neophobia occurred on Day 3, when the novel auditory background became familiar.

We repeated the above analysis for the *6OHDA* groups. There was a significant effect of *Day* [F(3,36)=83.85; p<.001] and no other effect or interaction (all *p's*>.2). Post-hoc analysis of the effect of *Day* using Bonferroni-corrected t-tests confirmed less vinegar intake on Day 1 compared Days 2, 3, and 4 (all *p's*<.005) but no other comparisons were significant. This indicates that unlike the sham rats, the animals injected with 6-OHDA attenuated the neophobic response to the vinegar taste on Day 2, regardless of whether the background tone was or was not changed.

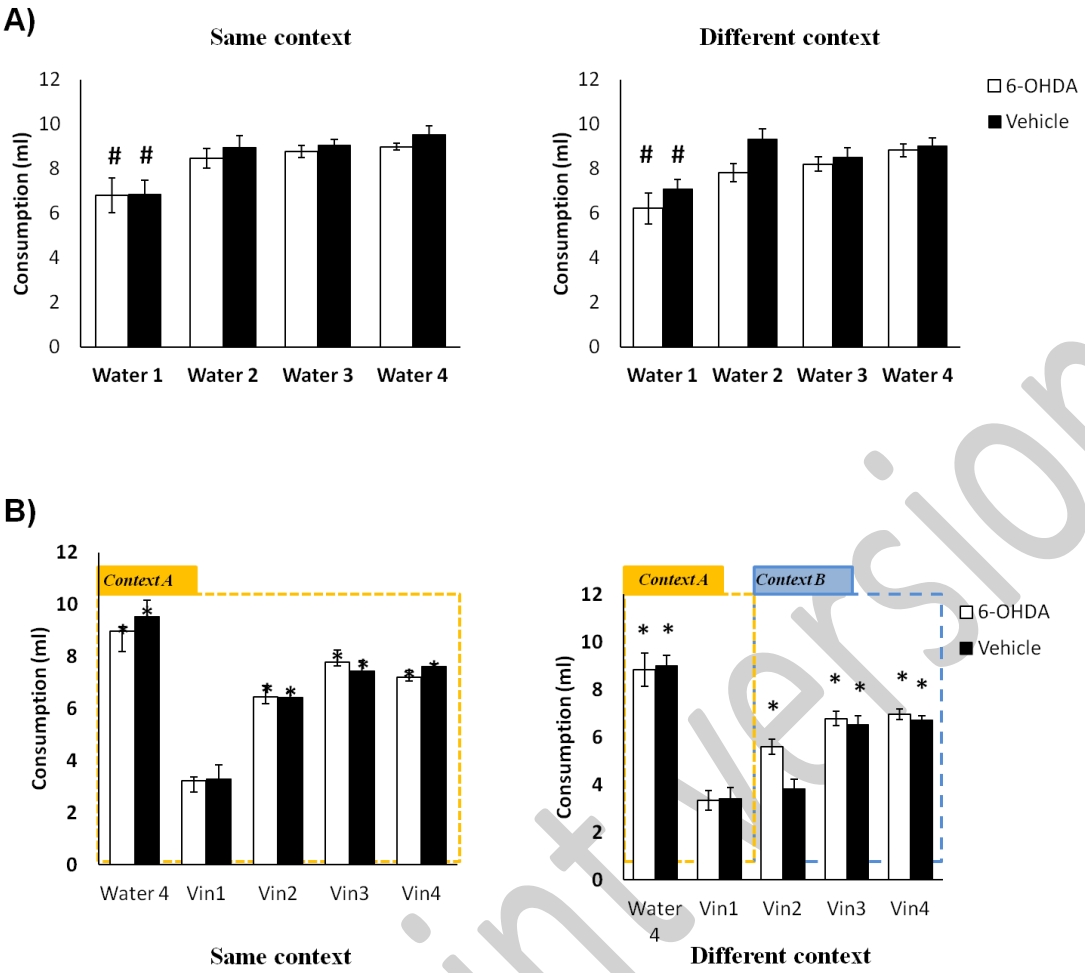
A One-way ANOVA that compared the intake of vinegar amongst the groups on the second day of vinegar was applied. There was a significant effect of group [F(3,28)=8.275; p=.004]. Further post-hoc analysis of the effect using Tukey´s multiple comparisons tests confirmed less vinegar intake of the Sham-Different group was lower than the rest of the groups (all *p´*s <.05) and no other difference was observed between the rest of groups.

Lastly, an additional 4 X 2 (*Day* X *Context Change*) repeated measures ANOVAs were performed for groups of rats that were exposed only to water. The analysis did not reveal any significant effect or interaction (all *p´*s>.05), showing that all the groups drank similar amounts of water regardless the change in the auditory background. An additional Student´s T-test comparing the amount of water consumption on day 4 (the day where the auditory background changed for the Different group), did not reveal any statistical difference in water consumption (t=.289; *p*=.78).

3. Immunofluorescence analyses.

Multiple independent samples T-tests comparing the number of DAPI stained cells between the Sham and 6-OHDA groups for the three regions of interest (dCA1, vCA1, vCA2, vCA3, DG and VTA) did not reveal any significant differences between Sham and 6-OHDA animals (all *p´*s >.8).

Multiple independent samples T-tests comparing the number of TH+ stained fibers between the Sham and 6-OHDA groups for the three regions of interest (dCA1, vCA1, vCA2, vCA3, DG and VTA) revealed a decreased TH labeling in all the ROIs in 6-OHDA animals compared to Sham (all p´s<.03), except for VTA regions (*p*=.76, respectively).



Supplemental Figure 1. A) Mean (±SEM) of the consumption of water during baseline of rats that were i.c. injected with 6-OHDA or vehicle. On the left panel the groups kept the auditory background constant. On the right the groups experienced an auditory background change on vinegar day 2. **B)** Mean (±SEM) of the consumption of vinegar during Phases I and II of the group that were exposed to the same context (on the left) and the group that experienced a change in the auditory background on vinegar day 2 (on the right). The symbol # represents statistically significant differences (p<.05) compared to Water 4 within the same injection group.