Supplementrary Materials for

Synergistic olfactory nerve input and cholinergic neuromodulation can activate ERK in rat olfactory bulb vasopressin cells

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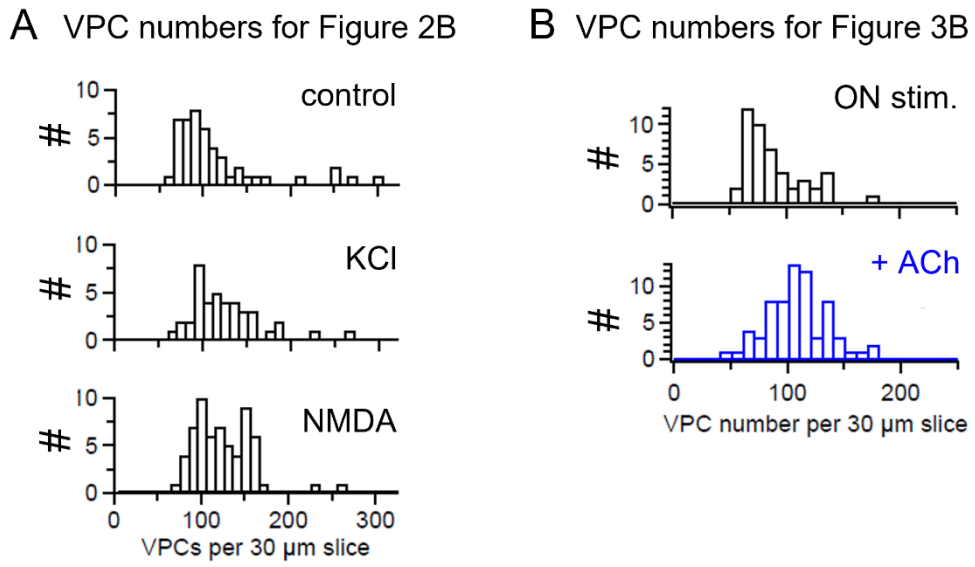

Supplementary Fig 1 Vasopressin cell numbers across conditions
Counts of VPCs in the respective conditions across 30-µm slices. **A** Control: 114 ± 55, n = 47 slices, KCl: 124 ± 41, n = 41 slices, NMDA: 124 ± 34, n = 62 slices **B** ON stimulation: 87 ± 26, n = 472 slices; ON stimulation + ACh: 107 ± 27, n = 68 slices

Supplementary Methods

Slice preparation

11-19 day-old juvenile rats of either sex were used for the experiments. The rats were deeply anesthetized with isoflurane and quickly decapitated. Horizontal slices (300 µm) were cut in ice-cold carbogenized ACSF (artificial cerebrospinal fluid; in mM: 125 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 20 glucose, 2.5 KCl, 1 MgCl, and 2 CaCl2) using a vibratome (VT 1200, LEICA, Wetzlar, Germany) and afterward incubated in ACSF at 36 °C for 30 min. The slices were stored at room temperature (~21 °C) in ACSF for 3 hours before any pERK-inducing assays (1) to reduce unspecific ERK activation due to the slicing procedure. Prior to electrophysiological recordings slices were stored for at least 1 hour.

Tetanic olfactory nerve stimulation

Brain slices were placed in a recording chamber as for the electrophysiology. Olfactory nerve (ON) stimulation was performed as described in Suyama et al. (2). Briefly, a glass pipette stimulation electrode was gently placed in the ON layer of the medial anterior OB inducing a stimulation paradigm after placement. The strength of a single stimulation pulse was 800 µA for 100 µs. Tetanic stimulation at 50 Hz was applied for 6 s, amounting to 300 pulses in total (three 1 s 50 Hz-trains spaced at 10 s were used

in Kawasaki et al. 2004). 10 min after stimulation, slices were transferred into a 12-well plate (Corning Incorporated, Corning, NY, USA) filled with 4 % paraformaldehyde in PBS and fixated at room temperature overnight. Then, PBS was exchanged for 30 % sucrose in PBS to cryoprotect the tissue for further slicing with a cryostat.

Pharmacology and chemical stimulation

The pharmacological agents (Sigma-Aldrich, Darmstadt, Germany) included acetylcholine (ACh) (100 µM as in 2), KCl (90 mM as in 1), and NMDA (100 µM as in 1). All drugs were diluted at the final concentration in ACSF for bath application during chemical or ON stimulation, or for wash-in during electrophysiological recordings.
For the ACh experiments, slices were perfused with ACh for 10 min before tetanic ON stimulation, and stimulation was performed in the presence of ACh.
KCl and NMDA stimulation ("chemical stimulation") were performed without tetanic ON stimulation. Slices were transferred into a small chamber with a mesh at the bottom. This chamber was incubated in a beaker filled with ACSF for 5 min, then it was transported into a beaker filled with KCl or NMDA and incubated for 5 min. Following chemical stimulation, the chamber was transferred back to an ACSF beaker and incubated for 10 min. Slices were then fixed with 4 % paraformaldehyde in PBS in the same manner as ON-stimulated slices (Figure 1A).

Cryo-cutting of stimulated slices

10 min after tetanic or chemical stimulation, slices were transferred into a 12-well plate filled with 4 % paraformaldehyde in PBS and fixated at room temperature overnight. Then, PBS was exchanged for 30 % sucrose in PBS to cryoprotect the tissue. To ensure permeation by the immunohistochemical reagents, 300-µm OB slices were cryo-sectioned into 30-µm slices (CM3050 S, LEICA, Wetzlar, Germany) at approx. -20 °C, following Kubota (3).

Labeling

All immunohistochemistry procedures were performed on 30 µm slices in 12-well plates, using the free-floating method (see Figure 1A). The staining protocol as previously described by Suyama et al. (2) was applied, using identical equipment and chemicals. In addition, slices were stained for DAPI using DAPI Fluoromount-G (SouthernBiotech).

Fluorescent microscopy

Fluorescent images of the stained 30-µm slices were obtained as previously described (2) using a DM6 B microscope driven by the software LAS X (LEICA). Z-stack pictures from approx. 6-7 different z-positions per 30-µm slice were used for analysis.

Cell counting

Immunoreactive VPCs were counted manually across the z-stack pictures of the 30 µm slices using the multi-point tool or cell counter plug-in in Fiji. Double-positive cells were identified by comparing two color channels for pERK (magenta) and GFP (green). DAPI staining (blue) confirmed the presence of a nucleus. The positions of counted cells were recorded. Due to difficulties with cryo-cutting, the number of recovered stained 30-µm slices per original 300-µm slice was variable. For inclusion in the analysis, at least two 30-µm slices per original thick slice containing at least 50 GFP-VPCs had to be recovered. Slices missing a large part of the OB were excluded from the analysis.

In tetanic ON-stimulated slices, immunoreactive cells were counted within all activated regions (denoted “activated”), and outside (denoted “outside”). Activated regions showed a band of $pERK^+$ MCs, often with columnar structures of $pERK^+$ GCs below. Borders of activated regions were delineated by the positions of the outermost $pERK^+$ MCs on either side and drawn perpendicularly to the MCL.
For the experiments following tetanic ON-stimulation or tetanic ON-stimulation combined with ACh wash-in, the numbers of $pERK^+$ MCs and $pERK^+$ GCs belonging to individual columnar structures were counted as well. Since it was not possible to also count $pERK^-$ MCs and GCs, these data could not be normalized. MCs and GCs were identified by their morphological appearance and their localization in the clearly defined MC and GC layers (4). MCs were counted manually. For GCs, we used a custom Fiji macro that automatically identified and counted $pERK^+$ cells within a certain region of interest (i.e. a column as defined above)
All pictures were analyzed by observers blinded with respect to stimulation groups.

VPC identification and electrophysiology
VPCs identification, whole-cell patch clamp recordings and their analysis were performed as previously described by (5). For identification and recording, a modified Zeiss Axioskop epifluorescence microscope (Carl Zeiss Microscopy, Oberkochen, Germany) with DIC illumination was used. Leaky cells with a holding current above ~ -10 pA were rejected. Experiments that showed a substantial drift in resting V_m were rejected. Pharmacological manipulations were performed via bath application of KCl (90 mM) or NMDA (100 µM), respectively. Traces were analyzed using IGOR Pro 6.37 (Wavemetrics, Portland, USA).

Fiji Macro, granule cell counting

```
// Duplicate and select copy
original = getImageID();
original_name = getTitle();
run("Duplicate...", "duplicate");
copy = getImageID();
copy_name = getTitle();
selectImage(copy_name);

// Bright & contrast
run("Brightness/Contrast...");
setMinAndMax(0, 15)
run("Apply LUT", "slice");
run("Close");

// ROI selection
run("ROI Manager...");
setTool("polygon");
waitForUser("Select, add and save ROIs");
roiManager("Save", "C:\Users\Nicolas Reichardt\Desktop\Roi.zip");

// New Table for results
table_name = original_name + "-Analysis";
Table.create(table_name);
setTool("hand");
```

```
// Smooth edges
run("Set Measurements...", "area display redirect=None decimal=3");
run("Smooth", "slice");
run("8-bit");

//Threshold (DEFAULT) and watershed
setAutoThreshold("Default dark");
run("Threshold...");
setThreshold(200, 255);
run("Convert to Mask", "method=Default background=Default");
run("Close");
run("Watershed", "slice");

// Particle analysis for each ROI
for (i=0 ; i<roiManager("count"); i++) {
    roiManager("select", i);

    // Measure and save ROI Area
    run("Measure");
    roi_name = call("ij.plugin.frame.RoiManager.getName", i);
    roi_area = getResult("Area");
    selectWindow(table_name);
    Table.set("ROI", i , roi_name);
    Table.set("ROI Area", i, roi_area);
    run("Clear Results");

    // Analyse particles
    run("Analyze Particles...", "size=10.00-200.00 circularity=0.30-
1.00 show=Overlay display exclude clear summarize slice");

    // Change Summary to Results to update our table with the data we
want
    selectWindow("Summary");
      IJ.renameResults("Summary", "Results");

      cell_count = getResult("Count");
      cell_area = getResult("Total Area");
      per_area = getResult("%Area");
      av_size = getResult("Average Size");

      selectWindow(table_name);
      Table.set("Cell count", i, cell_count);
      Table.set("Total Cell Area", i, cell_area);
      Table.set("%Area", i, per_area);
      Table.set("Average size", i, av_size);
}

selectWindow("Results");
run("Close");

// Save .xlsx using Read and Write Plugin - Read and Write Excel saves
the results table --> change Table name to Results
selectWindow(table_name);
Table.rename(table_name, "Results");

// Save Image
saveAs("Tiff", "C:\Users\Nicolas Reichardt\Desktop\tiff");

// Close all
```

```
waitForUser("Close all");
close("*");
selectWindow("Results");
run("Close");
roiManager("Delete");
```

References

1. **Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ, and Ji RR**. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *J Neurosci* 24: 8310-8321, 2004.
2. **Suyama H, Egger V, and Lukas M**. Top-down acetylcholine signaling via olfactory bulb vasopressin cells contributes to social discrimination in rats. *Commun Biol* 4: 603, 2021.
3. **Kubota Y**. Morphological and Neurochemical Characterization of Electrophysiologically Identified Cells. In: *Receptor and Ion Channel Detection in the Brain*, edited by Lujan R, and Ciruela F. New York, NY: Springer US, 2021, p. 353-382.
4. **Halász N**. *The Vertebrate Olfactory System: Chemical neuroanatomy, function and development*. Budapest: Akadémiai Kiadó, 1990.
5. **Lukas M, Suyama H, and Egger V**. Vasopressin Cells in the Rodent Olfactory Bulb Resemble Non-Bursting Superficial Tufted Cells and Are Primarily Inhibited upon Olfactory Nerve Stimulation. *eNeuro* 6: ENEURO.0431-0418.2019, 2019.