

Noxious stimulation alters neuronal activity in the ventral periaqueductal gray in a cell type-dependent manner

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List of abbreviations

Abbreviation	Description
5-HT	5-hydroxytryptamine (serotonin)
BA	Basal amygdala
BFR	Basal firing rate
BNST	Bed nucleus of stria terminalis
CeA	Central amygdala
CED	Cambridge Electronic Design
CRISPR	Clustered regularly interspaced short palindromic repeats
CS	Conditional stimulus
DA	Dopamine
DREADD	Designer receptors exclusively activated by designer drugs
DRN	Dorsal raphe nucleus
GABA	Gamma-aminobutyric acid
Glu	Glutamate
i.p.	intraperitoneal
NDS	Normal donkey serum
PAG	Periaqueductal grey
PB	Phosphate buffer
PE	Prediction error
PELT	Pruned exact linear time
PSTH	Peristimulus time histogram
RNA	Ribonucleic acid
SA	Streptavidin
TBS	Tris buffer solution
TH	Tyrosine-hydroxylase
US	Unconditional stimulus
VIP	Vasoactive intestinal polypeptide
Vglut	Vesicular glutamate transporter
vlPAG	Ventrolateral periaqueductal grey
vPAG	Ventral periaqueductal grey
VTA	Ventral tegmental area

Summary

The periaqueductal grey (PAG) is a midbrain structure incorporating the ventrolateral PAG (vlPAG) and dorsal raphe nucleus (DRN). These regions contain mainly four types of neurons: glutamatergic, GABAergic, dopaminergic, and serotonergic neurons. Neurons in the PAG are involved in controlling diverse brain functions including learning, sensorimotor activities, pain processing and autonomic processes. Malfunctioning of the circuit operation in the PAG contributes to several neuropsychiatric disorders, the treatment of which is still a great challenge. Therefore, understanding the functional properties of neurons in these regions can be critical in proposing new therapeutic approaches.

Using juxtacellular technique to record single cell activity in *in vivo* anesthetized mice in response to noxious stimuli, and post-hoc immunocytochemistry to identify the cell types, I was able to distinguish functionally different neurons in the PAG. Analysing the firing features and neurochemical content, I found that dopaminergic neurons can be separated into two groups based on their response latency and vasoactive intestinal polypeptide expression. Further, I revealed that serotonergic neurons are heterogeneous and can be clustered into five groups based on their responses upon noxious stimulation. These results uncovered a large functional heterogeneity even within the same groups of neurons, contributing to deeper understanding of network operation in this clinically important brain region.

Anatomical and functional organization of the ventral PAG

The ventral part of the PAG (vPAG) incorporates the vlPAG and the DRN, adjacent midbrain structures – located ventrally to the cerebral aqueduct – which regions are difficult to be spatially separated from each other by a clear border. In the following, I will provide a summary about the organization and aversive stimuli related functions of the two major neuromodulator systems in the vPAG, namely the serotonin (5-HT) and the dopamine (TH) system.

The serotonergic (5-HT) system

Approximately 30% to 50% of the neurons in the DRN are 5-HTergic, located in clusters (Steinbusch, 1981). Emerging evidence of anatomical, transcriptomical, and functional studies suggests a heterogeneous organization of DRN circuitry (Huang et al., 2019; Okaty et al., 2019). Based on specific marker genes, five subtypes of 5-HTergic neurons were distinguished that differ in numbers and special location, though there are some overlaps between the groups (Figure 1) (Huang et al., 2019). A distinct subtype of 5-HTergic neurons

in the ventromedial DRN expresses vesicular Glu transporter 3 (Vglut3) (Huang et al., 2019; Okaty et al., 2019), indicating that these neurons can co-release Glu (Liu et al., 2014).

In accordance with the complex function and organization of the vPAG, both inputs and outputs of this region are diverse, usually reciprocally innervated, and differ between spatially distinct 5-HTsub-types. To highlight some specifics, 5-HTsergic neurons are innervated by Gluergic neurons of the frontal cortical regions as well as multiple subcortical regions, whereas some other regions such as the CeA and the bed nucleus of stria terminalis (BNST) are sources of pure GABAergic inputs. Furthermore, local GABAergic and 5-HTergic inputs provide inhibitory modulation through GABA receptors and 5-HT_{1A} serotonin autoreceptors (Huang et al., 2019; Muzerelle et al., 2016; Weissbourd et al., 2014).

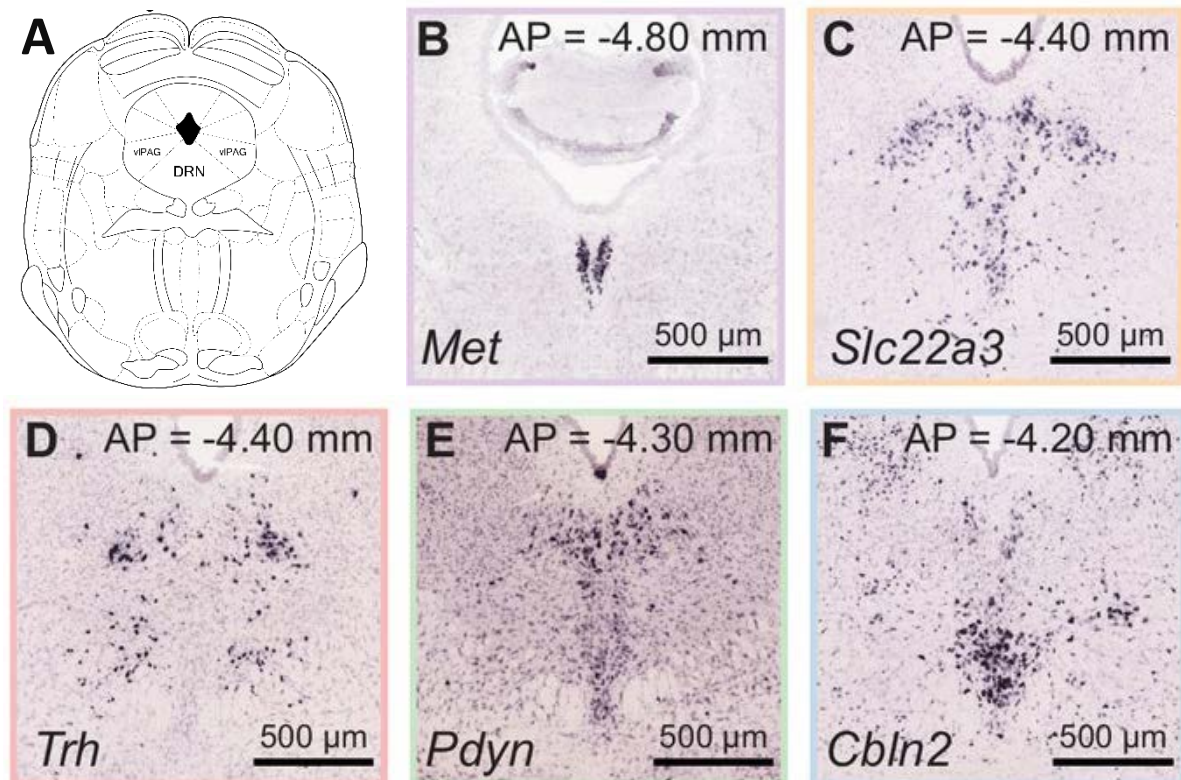


Figure 1. – 5-HTergic neurons subtypes form spatially distinct cell ensembles. **A)** Schematic figure of vPAG and DRN localization in the midbrain (AP=-4.6mm) **B-F)** Spatial distribution of 5-HTergic neuron subtypes, based on differentially expressed marker genes (Met, Slc22a3, Thr, Pdyn, Cbln2) Source of B-F: Huang et al., 2019

Aversive signal processing by 5-HT neurons in the DRN

Aversive signal processing has a critical role in learning and shaping behavioural responses. It was observed by several studies that 5-HTergic neurons of the ventral vPAG have a role in this process showing either enhanced or decreased neuronal activity on multiple timescales to aversive stimuli (Cohen et al., 2015; Ren et al., 2018; Schweimer & Ungless, 2010). However, the exact function of these neurons in aversion is still a subject to debate. It

was believed that increased 5-HT levels promotes waiting (Fonseca et al., 2015; Miyazaki et al., 2014), but contrary, applying selective serotonin re-uptake inhibitors – commonly used as antidepressants – reduce immobility (Seo et al., 2019). To resolve this contradiction researchers observed the population dynamics of the 5HTergic neurons in the DRN using fiber photometry in environments with different threat levels. They found a decreased activity of the 5-HTergic neurons upon movement in low- and moderate-threat situations, however the opposite was observed in a high-threat environment. In addition, photostimulation of 5HTergic neurons in the DRN, suppressed or promoted movement, depending on whether the anticipated threat was low or high (Seo et al., 2019). These results proposed that the 5HT system can switch between distinct operational modes in an environment-specific manner, contributing to the adaptive behaviour. Furthermore, 5-HT specific photostimulation in the DRN decreased mechanosensitivity in mice, suggesting a role in sensory signal regulation as well (Dugué et al., 2014).

Recent studies revealed multiple aversion processing pathways, which were 5-HT subtype specific. In the $DRN^{5-HT} \rightarrow$ orbitofrontal cortex pathway 5-HT expressing neurons showed an increased activity to rewards and punishments. Similarly, $DRN^{5-HT} \rightarrow$ CeA projecting neurons enhanced their activity to rewards, but oppositely responded to aversive stimuli. Using conditional Tph2 knockout experiments it was shown that $DRN^{5-HT} \rightarrow$ CeA pathway promote anxiety like behaviour in a 5-HT dependent manner (Ren et al., 2018). Another study uncovered a $DRN^{5-HT} \rightarrow$ basal amygdala (BA) pathway, which activity tracks fear acquisition, expression, and extinction. Bidirectional modulation of fear learning and extinction was possible using optogenetics. Excitation of the $DRN^{5-HT} \rightarrow$ BA pathway increased oscillation power – linked to fear states – and phasic-locked firing of BA neurons. Despite that the majority of 5-HTergic cells in the DRN express Vglut3, the usage of 5-HT_{A1} and 5-HT_{A2} receptor antagonist completely diminished the excitatory effect, which indicates the 5-HT dependency of this pathway in fear modulation (Sengupta & Holmes, 2019).

So far it was demonstrated that 5-HTergic neurons in the PAG play a role in variety of functions. Although these studies had two common drawbacks: they either observed population responses – thus disregarding the heterogeneity of the system – or inspect activity of single neurons in a particular task. This problem was solved in a recent study using one-photon Ca²⁺ imaging of 5-HTergic neurons with miniaturized microscopy across different behavioural tasks (Paquelet et al., 2022). First, the authors uncovered a hidden feature of 5-HTergic neuron responses besides the already known excitation and inhibition: during the repetition of a reward-

driven task approximately the 2/3 of the excited neurons showed adaptation in their activity. Second, the authors hypothesised that activity of 5-HT neurons reflected not individual stimuli, but rather a salience signal. To test the idea the neuronal activity was monitored in several independent conditions, such as gustatory stimuli with varying valence, foot shock and social interaction with a novel conspecific. In all the conditions a large set of 5-HTergic neurons altered their activity in response to stimuli – either to a predictive neutral stimulus – with either positive or negative valence, but not to neutral stimuli. Additionally, tracking the very same neurons over these behaviours revealed a mixed selectivity of 5-HTergic neurons, which means that a prominent proportion of neurons was similarly responsive regardless the type of task, excluding some extreme pairings (Paquelet et al., 2022).

In summary, the 5-HT system in the DRN is formed by multiple subtypes of 5-HTergic neurons, forming an extensive network that contributes to a wide variety of functions, including behaviours with emotional valence such as reward, aversion and social interaction processing, patience, sensory signal regulation and learning. Besides the defined pathways controlling individual functions, there are neurons with a mixed selectivity.

The dopaminergic (TH) system

DAergic neurons in the PAG are small in numbers (app. 1000 neurons in mice) which can be anatomically divided into two groups: the small, typically bipolar, fusiform cells encompassing the ventral part of the aqueduct; and the larger multipolar neurons, which are located ventrally compared to the small DAergic neurons (Figure 2). Studies using single cell RNA sequencing revealed that each DAergic neuron in the ventral PAG expresses the mRNA of vasoactive intestinal polypeptide (VIP) – which is presumably released as a neuromodulator in addition to DA. The VIP expression in DAergic neurons, however, varies. DAergic neurons expressing VIP at lower levels also express nicotinic receptors, which group can be further divided based on the expression of neurotransmission related genes (such as the gene of Vglut2) (Huang et al., 2019). These results propose a functionally heterogeneous DA system in the vPAG, however we still lack subtype specific studies, leaving the researchers with many open questions.

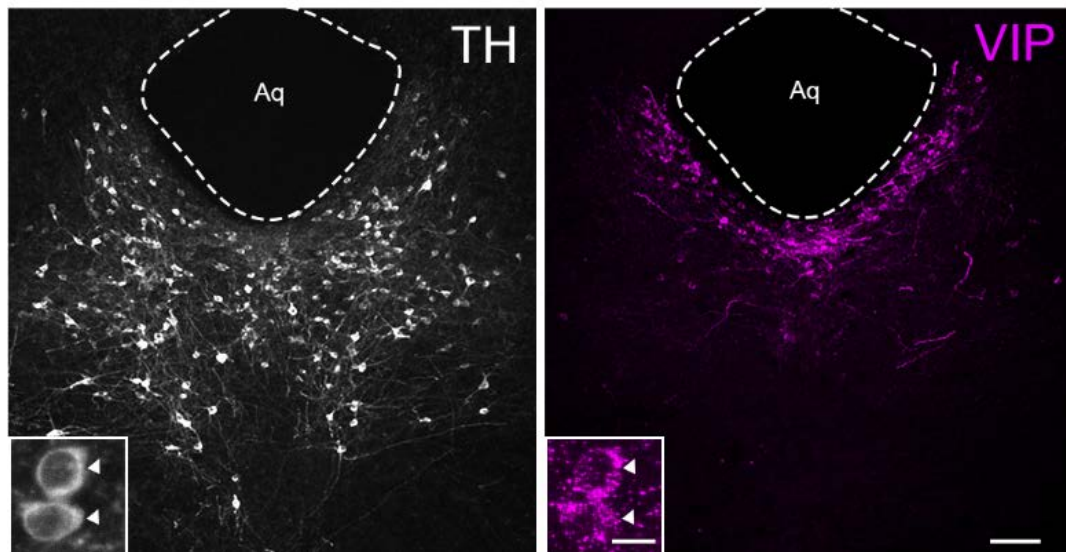


Figure 2. – Spatial distribution of TH⁺ and VIP⁺ neurons in the ventral PAG. Neurons were labeled using mouse anti-TH (TH⁺) and rabbit anti-VIP (VIP⁺). Scale bars are 100 μm and 10 μm.

Source: This figure and all corresponding work (immunohistology) was made by Kinga Müller.

DAergic neurons in this region mainly project to the two parts of the extended amygdala, namely the oval nucleus of the BNST and the lateral part of the CeA (Lin et al., 2020; Poulin et al., 2018; Zhao et al., 2022). Furthermore, these neurons also project to the paraventricular nucleus of the thalamus, the VTA, and nucleus accumbens, but in fewer numbers (S. Li et al., 2014). Inputs of these DAergic neurons are currently unknown.

Aversive stimulus related functions of DAergic neurons in the ventral PAG

DAergic neurons in the PAG are functionally heterogeneous. Several neuronal types in the PAG were implicated regarding nociception, including GABAergic, Gluergic and DAergic neurons, however in-depth investigations of the circuitry have uncovered striking differences from a therapeutic point of view. Manipulating both GABA and Gluergic neurons have an analgesic effect, but due to their connectivity (for example their inputs from the CeA) they also a part of circuits controlling anxiety like behaviours, both in mice and humans (Taylor et al., 2019). To probe whether DAergic neurons in the vPAG can have the same analgesic effect researchers expressed designer receptors exclusively activated by designer drugs (DREADD) in DAergic neurons exclusively in the vPAG. Excitation of these neurons decreased the sensitivity of the animals in thermal, mechanical, and inflammatory pain driven nociception, whereas inhibition increased their sensitivity. The analgesic effect size was similar to Gluergic neurons activation. To clarify whether the analgesic effect is purely DA driven, selective D1 and D2 antagonists were applied systematically, however only a nonselective DA receptor antagonist could eliminate the analgesic effect (Taylor et al., 2019). Animals treated with the

designer drug did not show any alteration during light/dark and open field test – two well-known behavioural tests to study anxiety like behaviour – in their behaviour compared to control mice. These data suggest an anxiety independent analgesic effect upon the activation of these DAergic neurons in the vPAG (C. Li et al., 2016; Taylor et al., 2019). A recent study revealed that BNST projecting DAergic neurons in the ventral part of the PAG (vlPAG/DRN^{DA} → BNST) are responsible for analgesic effect (Yu et al., 2021). Previous studies examined pain related functions of DAergic neurons in male animals exclusively. Using a similar DREADD approach as described before to excite DAerg neurons in the vPAG they compared the analgesic effect in female and male mice. Strikingly, they observed the expected analgesic effect in the male mice, but no effect in case of the female mice. Using optogenetic approach the sex-differential reduced nociception could be reproduced with the photostimulation of the vlPAG/DRN^{DA} → BNST axons. Stimulation of the vlPAG/DRN^{DA} → BNST during several different behavioural assays yielded with increased locomotor activity, which was female specific. Electrophysiological experiments revealed a difference in the strength of the functional connectivity regarding the vlPAG/DRN^{DA} → BNST pathway, namely female mice possess less polysynaptic inhibition, while male mice showed increased D1 receptor gated depolarization than females, strengthening the notable differences between the two sexes (Yu et al., 2021). These results proposed a sex specific role of DAergic neurons in the vPAG in nociception and locomotion.

One prominent target area of the DAergic neurons in the vPAG is the CeA, a part of the amygdala nuclei, which are essential for fear memory formation. The CeA receives the major DAergic input from the vPAG, and the circuitry of the CeA are reciprocally interconnected with this DAergic system, suggesting a fear-related function of the region (Groessl et al., 2018). Groessl et al. found that the responses of DAerg cells in the vlPAG shifted from the unconditioned stimulus (US) to the conditioned stimulus (CS) during conditional learning, which suggests that these cells differentiate between the expected- and the real event, thus they encode prediction error (PE), like other non-DAergic cells in the ventral part of the PAG (Ozawa et al., 2016). Moreover, they found that these DAergic cells positively encoded unexpected aversive stimuli. Such aversive PE signals were also detected in humans (Roy et al., 2014). According to hypothesis of Groessl et al., the DAergic cells in the vPAG provide the teaching signal, which changes according to the predictive value of the CS, and then the CeA converts this signal into a fear memory trace (Groessl et al., 2018). In addition, negative feedback from the CeA to the DAergic cells in the vPAG has been identified, which can adapt

the PE signal, depending on the activity of the CeA. If these DAergic cells encode a PE-linked reinforcement signal, then their activity should regulate learning during different associations. The researchers found that optogenetic inhibition of DAergic cells in the vPAG during the CS-US pairing - thereby simulating that the value of PE is lower than in reality - resulted in a reduced CS-US association, while in the opposite case, when these cells were activated during the same conditions, facilitated the association of the new CS with the US, thus supporting the role of these cells in associative fear learning (Groessl et al., 2018).

DAergic cells of the vPAG responded at the population level to any salient stimuli, regardless of whether the stimulus valence is positive or negative (Cho et al., 2017, 2021). Additionally, a subset of these neurons responded to the conditional stimulus after fear/reward learning faithfully, but in a context dependent manner (for example this was observed in freely moving mice, but not in head-fixed animals) (Cho et al., 2021).

A recent study expended the two former hypotheses, which includes both valence independent stimulus signalling and fear-related functions of the DAergic system in the DRN, proposing that these neurons control the expression of incentive memories (Lin et al., 2020). They found that inhibition of DAergic neurons in the DRN during memory expression – but not during conditioning – in both reward-driven conditional place preference and fear conditioning tasks, decreased the animal performance and freezing, respectively. To prove that memory expression is DA dependent, researchers used virus mediated CRISPR/Cas9 technology to down-regulate the TH expression in vivo. This method effectively attenuated freezing time and conditional place preference, which suggest that DA has a key role in incentive memory expression (Lin et al., 2020).

In summary DAergic neurons in the PAG can be further distinguished by their VIP expression and mainly project to the extended amygdala. This system plays a role in clinically relevant functions such as fear-learning and nociception, but the exact mechanisms of these functions are not well understood.

Objectives

Our objective was to determine i) the changes of neuronal activity *in vivo* in response to painful stimuli at a single-cell resolution in the vPAG and ii) to reveal the types of neurons based on their neurochemical content. Combining immunocytochemical and electrophysiological data we assessed whether functionally distinguishable subgroups exist. We propose that our results will help to understand the functional complexity of midbrain neuromodulator systems.

Materials and Methods

Animals

In these experiments adult mice were used with C57Bl/6J or B6129F1 genetic background and varying age (mean=123±45.54) which were housed under 12-h light/dark cycle with free access of food and water.

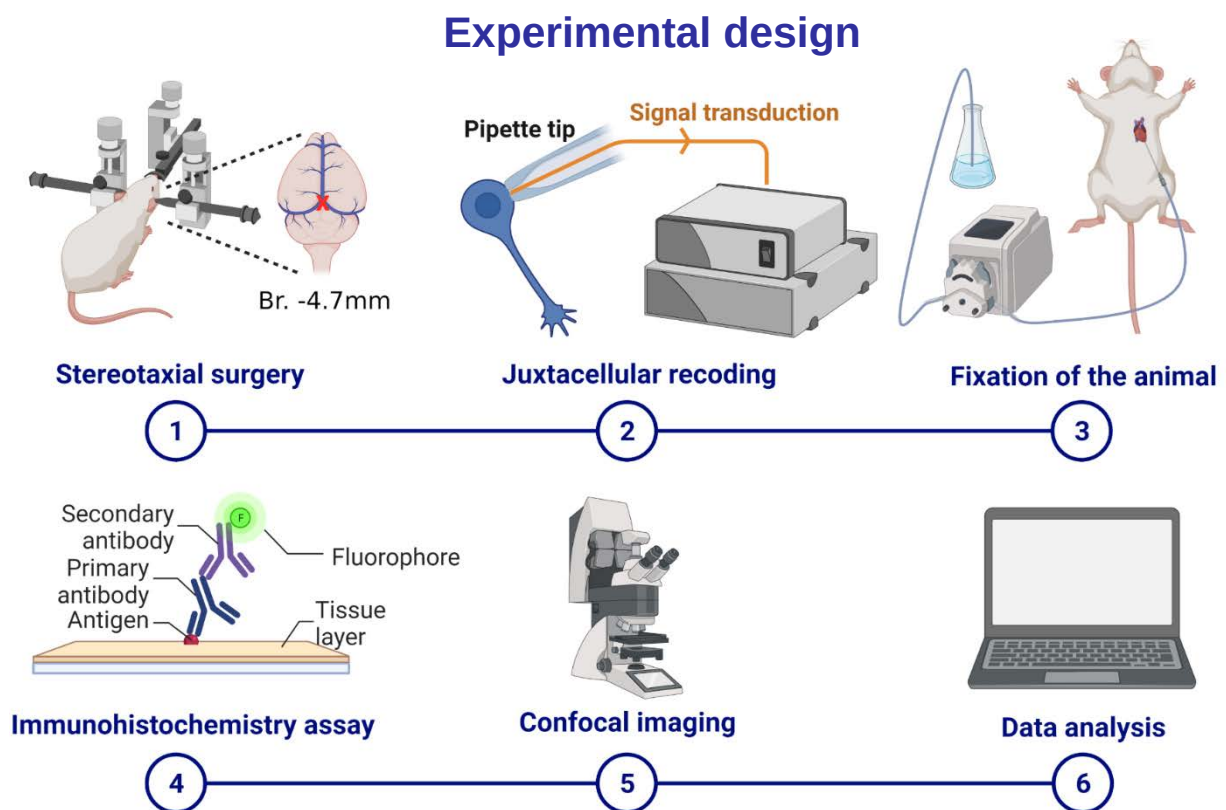


Figure 3. – Graphical abstract of the experimental procedure
Created with [BioRender.com](https://www.biorender.com/).

Stereotaxic surgery

Mice were anesthetized with isoflurane (2%) carried by O₂ (at a rate of 0.4 l/min) and then placed in a standard stereotaxic instrument. Before any incision lidocaine was applied on the scalp, locally. The animal's temperature was continuously maintained using a heating pad.

After several preliminary experiments, appropriate coordinates were determined at 4.7 mm posterior to the bregma, in the midline of the sagittal plane, to hit the area with the highest number of VIP+ cells. Craniotomy was conducted with a great caution due to exposure of the *confluens sinuum*. Next, the headplate was fixed to the skull horizontally using dental cement to increase stability and accuracy, while leaving the target area approachable. After the surgery, 0.15 ml of 20% urethane intraperitoneal (i.p.) was injected to mice and after a few minutes the recording session has started.

Electrophysiological recording

Throughout the recording session the appropriate body temperature of the animal was maintained with a heating pad, and, if necessary, additional 0.05 ml of 20% urethane was injected i.p.. First, the animal was placed into the setup by firmly securing the headplate into the holder, which provided a stable, but painless fixation of the head. For juxtacellular recordings (Pinault, 1994), a glass micropipette was fabricated with a standard vertical puller, from borosilicate capillaries. The micropipette – the resistance of which fell in the range of 18-30M Ω – was filled with 3% Neurobiotin solution (Vector Labs, dissolved in 0.5M NaCl), and then an Ag/AgCl wire was dipped in the intrapipette solution. The Ag/AgCl wire was connected to the main amplifier (MultiClamp 700B) via a headstage (CV-7B). A reference electrode was placed under the skin of the animals' neck. For stimulation, two metal conductors were attached to both of the animal's legs that transmitted a weak current generated by the BioStim (SuperTech Ltd), causing a mild electric shock. Noise cancelling (50Hz) was done using a Hum Bug filter. Analog signals were digitalized using a Cambridge Electronic Design (CED) 1401 A/I type analog-digital converter. Since approaching the target area (2900-3400 μ m deep from the vessel surface) requires to go through the *confluens sinuum* – which could break the glass micropipette or cause serious bleeding – slow advancement (1-2 μ m/s) with micropipette to the brain was required, which prevented bleeding and caused the brain tissue to retain its original shape, providing a more stable recording. If action potentials with appropriate amplitudes (>0.4 mV) were registered in the target area, a conductivity increasing gel (Rextra) was applied between the metal conductors and the skin. Monitoring the base firing activity of the neuron lasted for a few minutes and then a protocol applying foot shocks was executed, while continuous recordings of the electrical signals were maintained. The 'shock protocol' consisted of 40-80 mild electric foot shocks (3ms, ~ 1 mA), which were delivered alternately between the paws in every 15 s. To fill the cell with Neurobiotin solution iontophoretic, positive nanocurrents as square signals were delivered, gradually increasing the current from 100pA

until the cell showed current-modulated firing. Upon achieving the firing modulation, this state was maintained as long as possible, but usually no more than 20 minutes and then the micropipette was retreated. Depending on the anaesthesia and the quality of the recording, the whole recording process was restarted with a minimum of 150µm change in the anterior-posterior or dorso-ventral axis to be able to distinguish the labelled neurons.

Histology

Animals were perfused under deep anaesthesia directly through the circulatory system (Gage et al., 2012) with 0.9% saline for a few minutes followed by 120-150ml, 4% paraformaldehyde fixative in phosphate buffer (PB, 0.1M, pH=7.4). After the removal of the brain, it was placed in PB or fixative for at least 12 hours, depending on whether the perfusion was sufficient. Next, 40µm or 150µm sections were cut using a vibratome (Leica) and washed for 3x15 minutes in PB followed by washing in tris buffered saline (TBS) for another 3x15 minutes. For staining sections were incubated for approximately 15 hours in solution containing Cy3-conjugated Streptavidin (SA, 1:20.000 in TBS, Sigma-Aldrich). Next, sections were mounted onto slides in VectaShield (Vector Labs), cover slipped and analysed under an epifluorescent microscope (Axioscope, Zeiss). Because of limited penetration of specific antibodies cell body containing 150µm-thick sections were first re-sectioned to 40µm by Kinga Müller and then incubated in blocking solution containing 10% normal donkey serum (NDS), 0.5% Triton-X (10%) dissolved in 0.1M PB for 30 min. Next, these sections were incubated in primary antisera developed against 5-HT (rat anti-5-HT, 1:500, in 1:2 glycerol, Millipore), TH (chicken anti-TH, 1:2000, in 1:2 glycerol, ImmunoStar), and VIP (rabbit anti-VIP, 1:2000, in 1:10 PB, ImmunoStar) for 24 h at room temperature and 4 days at 4°C. Then, sections were washed for 3x20 minutes in PB and stained with Alexa488 (donkey anti-rat, 1:500, Molecular Probes), Alexa647 (donkey anti-rabbit, 1:500, in 1:2 glycerol, Jackson), DyL405 (donkey anti-chicken, 1:500, in 1:2 glycerol, Jackson), and 1% NDS containing solution for 3 hours. After 3x10 minutes washing in PB, sections were mounted onto slides and analysed using a confocal microscope (Nikon Ni C2 Confocal Microscope, Z-stacks with 1µm step size). For presentation purposes images were modified using Image-J FIJI.

Data analysis

Cell-type classification was based on visual inspection of confocal images by two independent researchers. In cases when the labelled cell could not be corresponded confidently with electrophysiological recording or if cell-type identity was questionable, the data was excluded from further analysis. Electrophysiological data and timestamps of shocking were

captured using Spike2 (Cambridge Electronic Design) at a sampling rate of 20 kHz. Next, data was imported into a custom-made Python environment – comprising 3rd party packages –, where it was pre-processed and analysed in a custom-written pipeline (more than 1500 lines of code written by myself). Averaged spike waveforms were manually analysed using their initial, first and second derivate forms. Basal firing rate (BFR) was calculated as an average of spike counts during a 100 second period before the first stimulus. To examine the effect of the foot shocks on the firing activity of neurons, data were binned and plotted as a peristimulus time histogram (PSTH) or a Z-score histogram. To find changes in the firing, mean-drift based changepoint detection was used (PELT searching method, Killick et al., 2011) with fine-tuned parameters. Spikes present between the changepoints (i.e. putative response time windows) were counted and were compared with the pre-stimulus spike count in multiple corresponding time windows. Statistically significant changes (Wilcoxon signed-rank test with discarding all zero-differences, $p < 0.05$) were accepted as stimulus-induced changes, which was used to cluster neurons into response classes. For comparisons of response latencies and intensities, non-parametric statistical tests, such as Kruskal–Wallis H-test with Dunn’s comparison ($N_{\text{groups}} > 2$) or two-sided Mann-Whitney *U*-test ($N_{\text{groups}} = 2$) were used. Neurochemically non-identified neurons were excluded from statistical comparison, since this group may involve multiple types of neurons.

Results

To determine the characteristics of changes in firing activity upon painful stimuli in vPAG neurons, I used the juxtacellular recording and labelling technique in head-fixed, anesthetized mice, which allowed us to reliably identify neuron types based on their neurochemical content. Mice received mild electric foot shocks (3ms, 1mA) at constant time intervals during the recording, serving as salient painful stimuli widely used in aversive conditioning paradigms. After successful recordings, neurons were filled with Neurobiotin. From all the recording attempts ($N_{\text{attempts}} = 175$), I was able to label a significant proportion of the recorded neurons (60%, $N_{\text{labeled}} = 105$). In the following, I will present data from successfully identified neurons ($N_{\text{cells}} = 79$, from $N_{\text{females}} = 30$, $N_{\text{males}} = 17$), which were located in the vlPAG or DRN, exclusively (Figures 4-5).

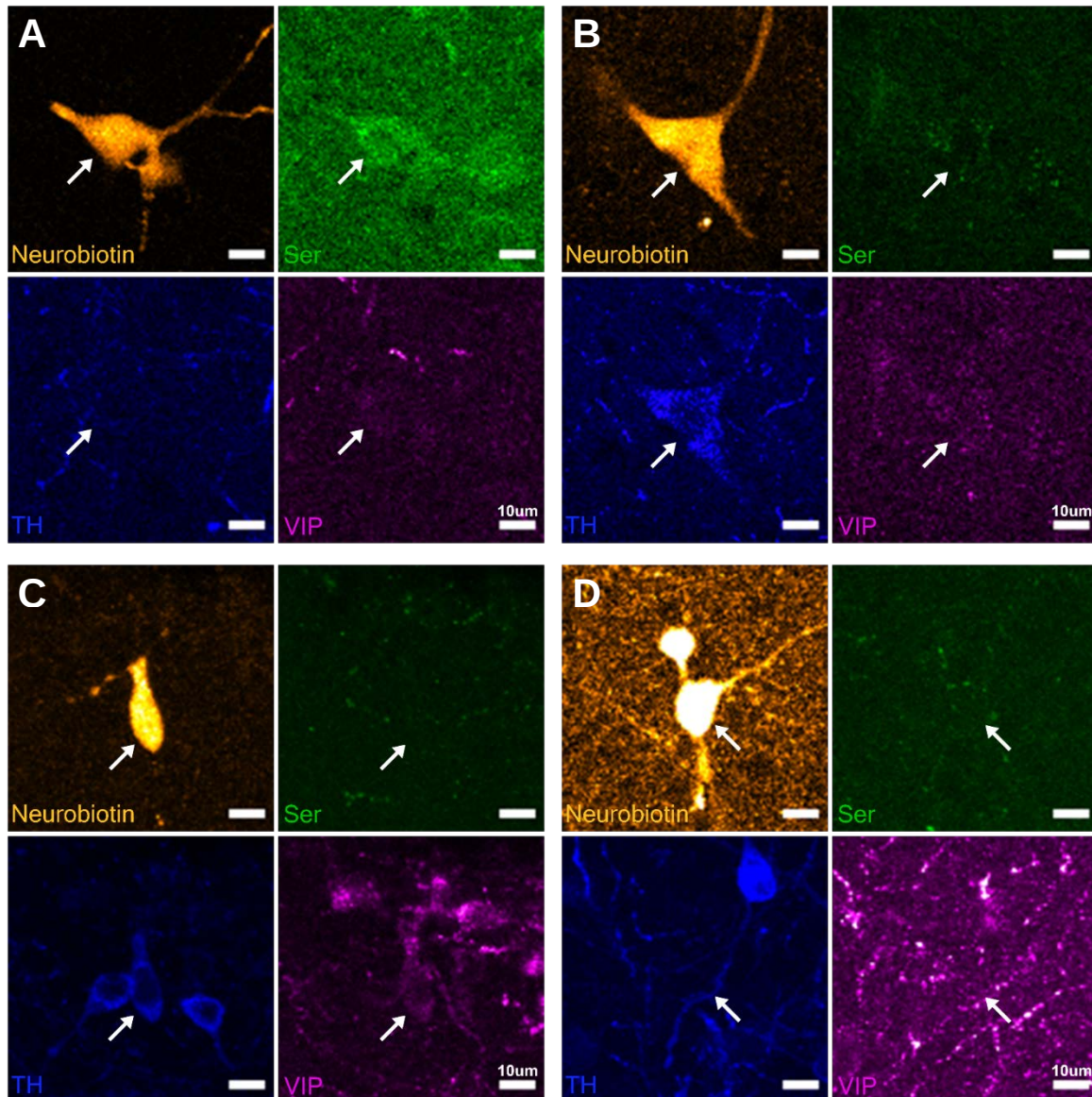


Figure 4 – Neurobiotin filled neurons in the vPAG can be reliably identified based on their neurochemical content. Images were captured with a confocal microscope at 20x magnification. For presenting purposes single images were chosen from z-stacks (1µm). **A-D)** Representative examples for 5-HTergic (Ser), DAergic (TH), VIP+ DAergic, and neurochemically non-identified neurons, respectively.

VIP+ neurons tend to have lower BFR compared to other neurons in the vPAG

Firstly, I separated the cells by their neurochemical properties, such as 5-HT, TH and VIP expression, forming 4 groups [5-HT+, TH+VIP-, TH+VIP+, and neurochemically not-identified ($\emptyset$)] (Figure 4). The proportion of the labelled neurons roughly represents the cell type distribution in this region, namely that 5-HTergic cells are more abundant in numbers, than DAergic cells (Figure 5). However, one of my main goals to reveal the features of VIP+ neurons, which are preferentially located in the close vicinity of the ventral part of the aqueduct

between the 3rd and 4th ventricle. Because of this bias in the labelling method, there is an overrepresentation in the number of TH+ and TH+VIP+ neurons.

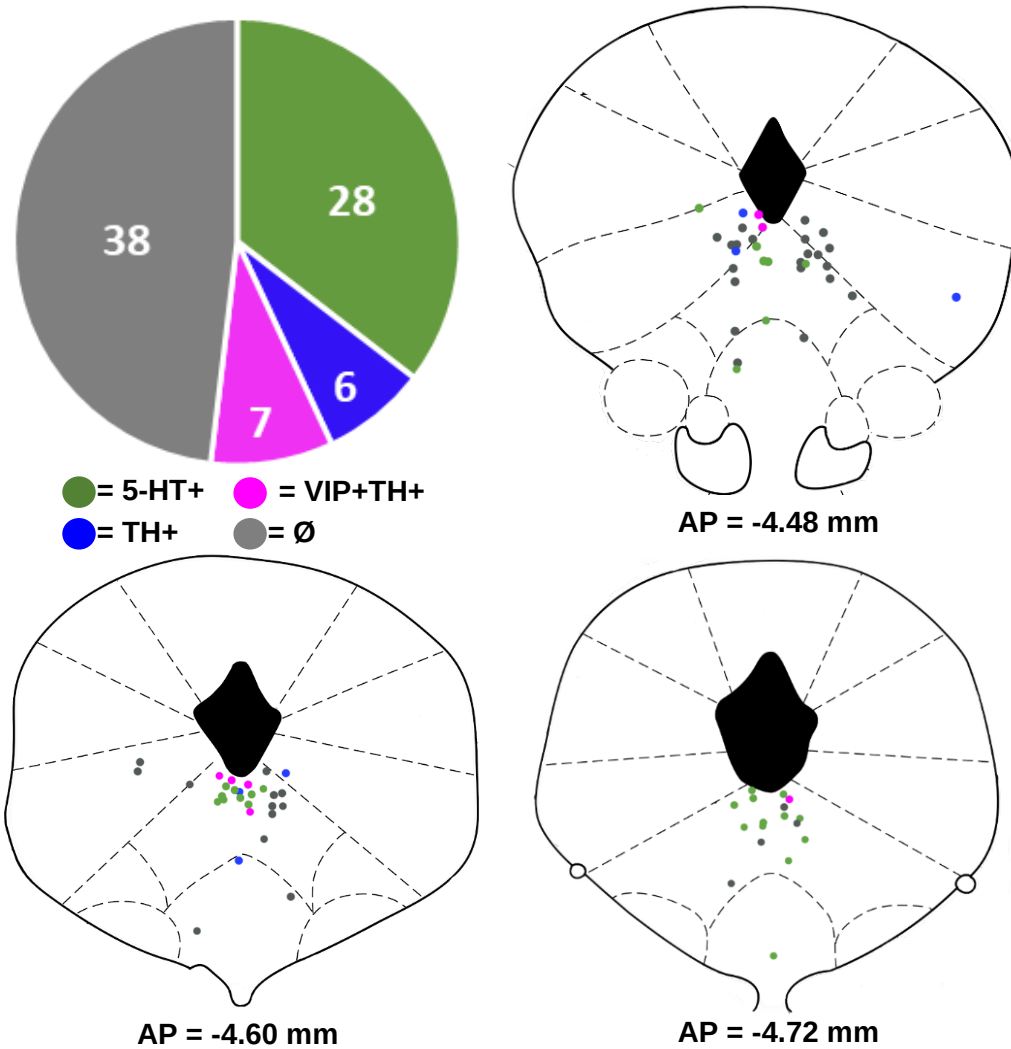


Figure 5. – Neurochemically identified neurons and their localization in the vPAG. VIP+ neurons are preferentially found in the close vicinity of the aqueduct as described previously.

Next, I examined the basic firing properties of the neurons, such as the BFR and spike waveforms. The analyses of the BFR uncovered that TH+VIP+ neurons have a lower firing rate (mean=0.46±0.30Hz, Kruskal–Wallis H-test, $p=0.023$) than other types of neurons in the vPAG/DRN and show significantly lower BFR compared to 5-HT+ neurons (Kruskal–Wallis H-test with Dunn’s comparison, $p=0.004$, Figure 6).

Regarding the spike waveforms, I took the average waveform samples from 23 neurons and compared ($N_{5-HT+}=10$, $N_{\emptyset}=6$, $N_{TH+}=4$, $N_{TH+VIP+}=3$), but found no cell-type characteristic spike waveforms (Figure 7). This might be e.g. due the nature of the juxtacellular recording technique, which detects spikes extracellularly, thus the recording electrode location – compared to the location of action potential generation – can influence the shape of the recorded spikes.

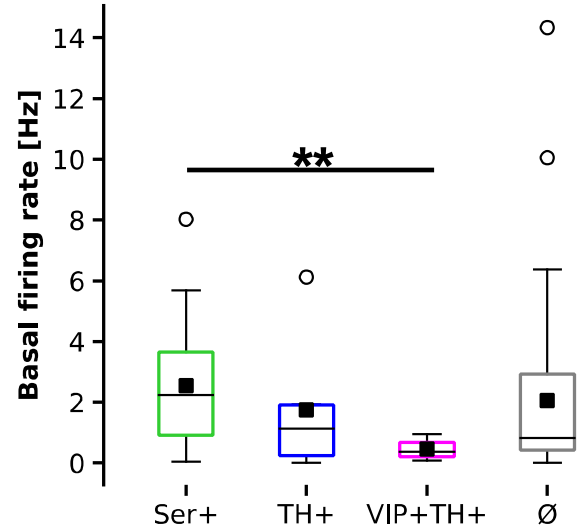


Figure 6. – VIP+TH+ neurons fire at significantly lower rate in basal conditions than 5-HTergic (Ser+) neurons.

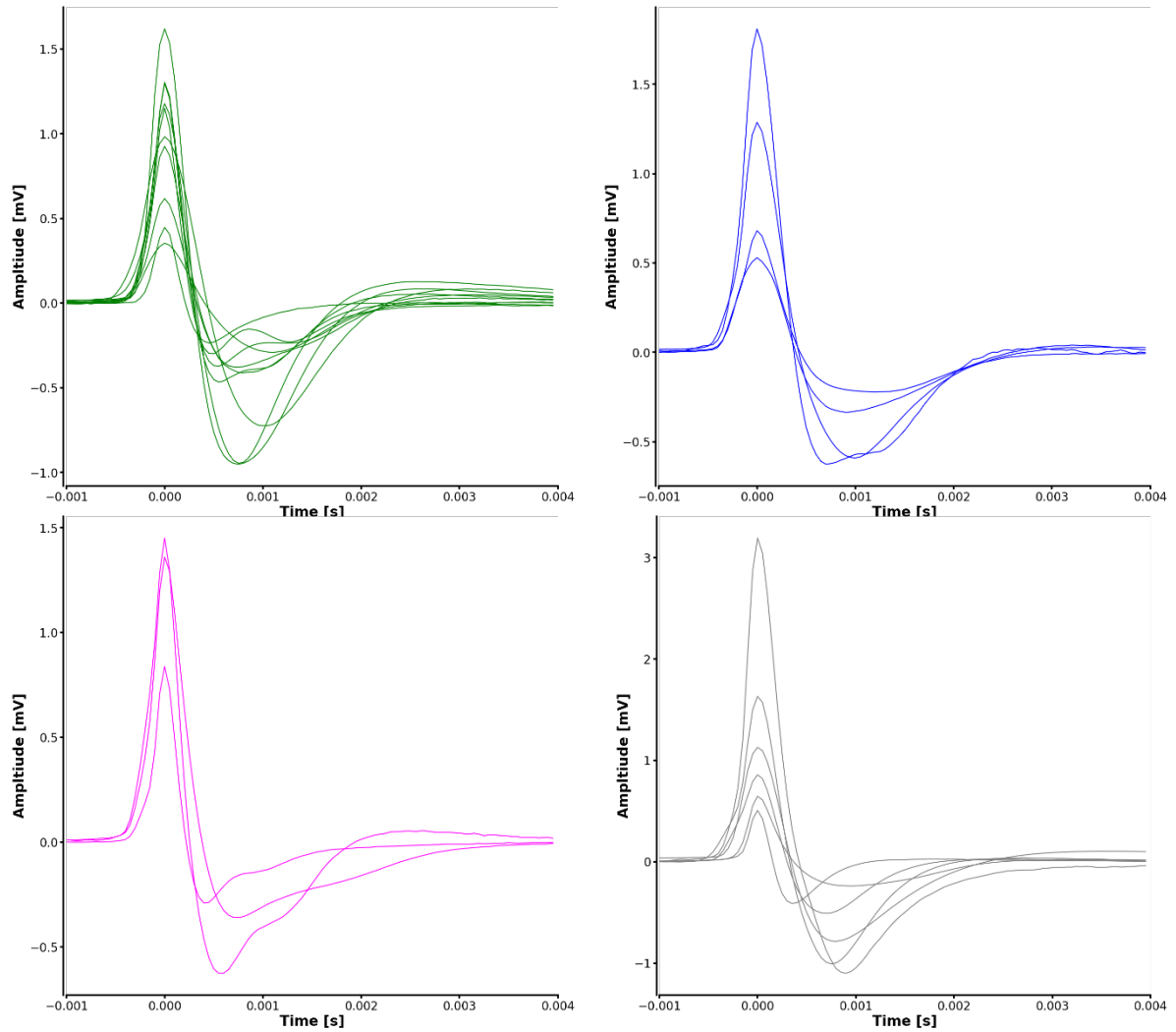


Figure 7. – Neurons in the vPAG cannot be distinguished by their waveform using juxtacellular recording technique.

Neuronal responses in the vPAG to noxious stimuli is cell-type dependent

After the analysis of PSTHs and Z-score histograms of the recorded neurons in response to foot shocks, I clustered the neurons into 5 classes based on the change in their firing activity upon stimulation: neurons that showed excitation (exc), neurons showed inhibition after their excitation (exc-inh), neurons showed inhibition (inh), neurons showed excitation after their inhibition (inh-exc) and finally neurons that did not respond to the stimuli (Figure 8). These results revealed the heterogeneous response of 5-HT⁺ and neurochemically non-identified neurons, whereas TH⁺ and TH+VIP⁺ neurons are primarily showed excitatory responses to noxious stimuli. However, these results raised the question: is there any difference between the same types of responses giving rise by different cell types? To examine this question, response intensities (i.e. changes in the Z-score magnitude) and time-windows were determined (see Materials and methods) and compared with each other for every cell type (Figure 9). Comparison of excitatory response intensities revealed no significant difference between the cell types (Kruskal–Wallis H-test $p=0.21$), but 5-HT⁺ cell showed significantly stronger inhibition compared to neurochemically non-identified cells (two-sided Mann-Whitney U test, $U=7.0$, $p=0.042$). Regarding the temporal dynamics of the responses, I found a statistically significant difference between the response latencies of different types of neurons (Kruskal–Wallis H-test, $p=0.009$), where TH+VIP⁺ neurons show excitatory responses at a later time point compared to other types of neurons (Dunn’s comparison Ser⁺ vs TH+VIP⁺ $p=0.005$; TH+VIP⁻ vs TH+VIP⁺ $p=0.006$). This founding suggests a functional difference between the two DAergic neuron types located in the vPAG. The length of these excitatory responses were similar (Kruskal–Wallis H-test, $p=0.21$).

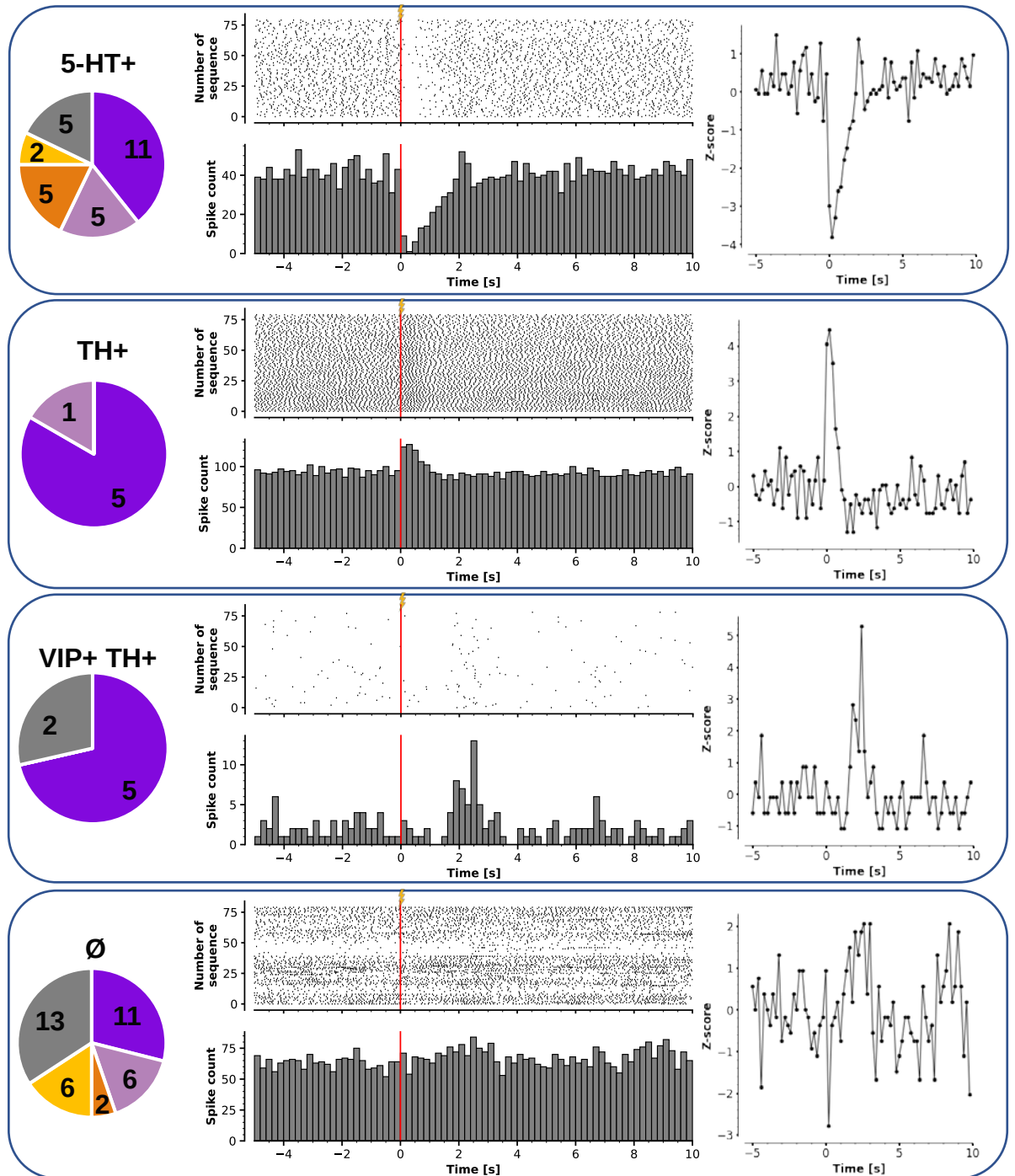


Figure 8. – Cell-type dependent responses to noxious stimuli in vPAG neurons with example PSTHs and Z-scores. Pie charts represents how different neurons responded to the stimuli. Majority of neurons showed excitation (purple) or biphasic excitation-inhibition (pink), whereas some of the 5-HT+ and neurochemically unidentified neurons responded with inhibition (orange) or biphasic inhibition-excitation (yellow). Some neurons did not alter their activity (grey). Bin size on PSTHs and Z-scores was set to 200ms. Red lines represent stimulus timings. The example 5-HT+ neuron was inhibited, the TH+ VIP- and VIP+ TH+ neurons were excited, whereas the unidentified neurons showed no change in its firing.

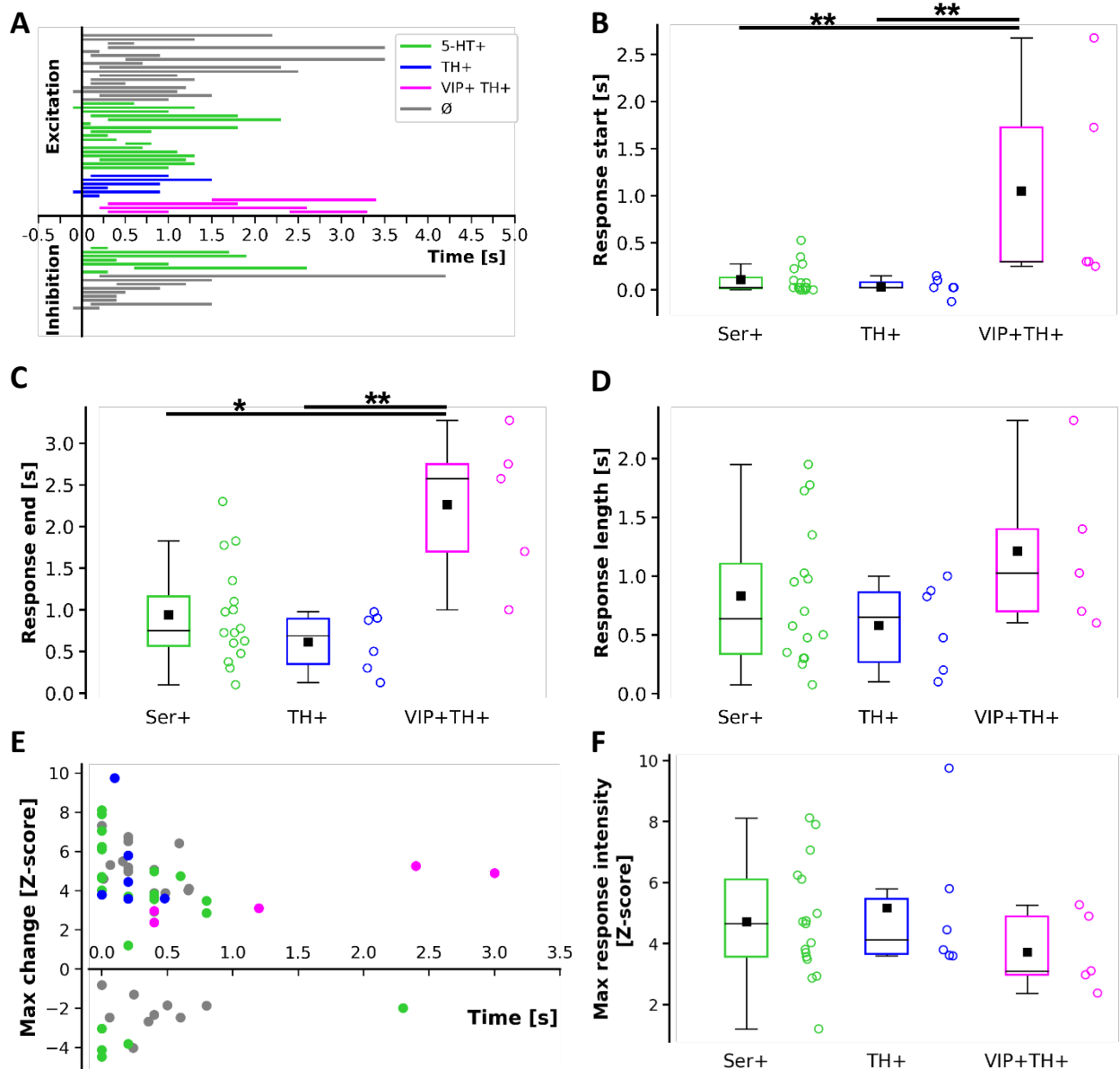


Figure 9. – VIP+TH+ neurons differ in their excitatory response from other types of neurons in the vPAG. **A)** Response time-windows of individual neurons. **B)** Response length of primer excitatory neuronal responses. **C)** Response latencies of excitatory responses. **D)** End timing of excitatory responses. **E-F)** Maximal change in Z-score values representing the intensity of responses.

Discussion

Here, I presented the heterogeneity of neurons in the vPAG regarding their neurochemical expression and their response to noxious stimulus. I distinguished 2 types of DAergic neurons: one expressing VIP – at levels detectable by immunohistochemistry –, and one without this neuropeptide. Both DAergic subtypes showed excitatory responses to foot shocks, but the firing elevation of VIP+ neurons occurred significantly later. Additionally, these

VIP+ DAergic neurons tend to have lower BFRs than other neurons in this area. These results suggest a functional difference between the two types of DAergic cells.

Functional diversity of 5-HTergic neurons of the DRN was recently described by other studies using a Ca^{2+} imaging technique, uncovering two types of responses (inhibition or excitation) at a single cell level with mixed selectivity (Paquelet et al., 2022). However, the juxtacellular recording technique provides better temporal resolution than Ca^{2+} imaging. I revealed – in addition to excited and inhibited 5-HT+ neurons – two more biphasic response types for these cell type. Moreover, the juxtacellular recordings can yield additional details, such as revealing the morphological features of the recorded neurons, including their projection, which will be a subject of future investigations.

In summary, our results extended the current knowledge about the midbrain 5-HTergic and DAergic neurons, by revealing a heterogeneity that may have a role in neuronal computation performed by these neuron types linked to challenging situations.

Author Contribution

Péter Földi:

- Stereotaxic surgeries, juxtacellular recordings, perfusions and slicing (all the 150 μm and ~30% of 40 μm slicing) were exclusively done by myself (175 recording attempts, from more than 70 mice).
- Streptavidin immunostaining of Neurobiotin labelled neurons and 75% (58 cells) of VIP, TH, and 5-HT immunostainings were done by myself.
- All the data analysis were done by myself using my own custom written Python scripts.
- My work uncovered an additional, so far unknown heterogeneity in neurons of the PAG regarding their response to noxious stimuli.

Kinga Müller (PhD-student):

- Slicing of 150 μm sections to 40 μm were done by Kinga Müller (56 cells).
- In case of 21 cells (25%) VIP, TH, and 5-HT immunostainings were done by Kinga Müller.

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References

- Cho, J. R., Chen, X., Kahan, A., Robinson, J. E., Wagenaar, D. A., & Gradinaru, V. (2021). Dorsal Raphe Dopamine Neurons Signal Motivational Salience Dependent on Internal State, Expectation, and Behavioral Context. *Journal of Neuroscience*, 41(12), 2645–2655. <https://doi.org/10.1523/JNEUROSCI.2690-20.2021>
- Cho, J. R., Treweek, J. B., Robinson, J. E., Xiao, C., Bremner, L. R., Greenbaum, A., & Gradinaru, V. (2017). Dorsal Raphe Dopamine Neurons Modulate Arousal and Promote Wakefulness by Salient Stimuli. *Neuron*, 94(6), 1205–1219.e8. <https://doi.org/10.1016/j.NEURON.2017.05.020>
- Cohen, J. Y., Amoroso, M. W., & Uchida, N. (2015). Serotonergic neurons signal reward and punishment on multiple timescales. *ELife*, 4(4). <https://doi.org/10.7554/ELIFE.06346>
- Fonseca, M. S., Murakami, M., & Mainen, Z. F. (2015). Activation of Dorsal Raphe Serotonergic Neurons Promotes Waiting but Is Not Reinforcing. *Current Biology*, 25(3), 306–315. <https://doi.org/10.1016/j.CUB.2014.12.002>
- Gage, G. J., Kipke, D. R., & Shain, W. (2012). Whole Animal Perfusion Fixation for Rodents. *JoVE (Journal of Visualized Experiments)*, 65, e3564. <https://doi.org/10.3791/3564>
- Groessl, F., Munsch, T., Meis, S., Griessner, J., Kaczanowska, J., Pliota, P., Kargl, D., Badurek, S., Kraitsy, K., Rassoulpour, A., Zuber, J., Lessmann, V., & Haubensak, W. (2018). Dorsal tegmental dopamine neurons gate associative learning of fear. *Nature Neuroscience* 2018 21:7, 21(7), 952–962. <https://doi.org/10.1038/s41593-018-0174-5>
- Huang, K. W., Ochandarena, N. E., Philson, A. C., Hyun, M., Birnbaum, J. E., Cicconet, M., & Sabatini, B. L. (2019). Molecular and anatomical organization of the dorsal raphe nucleus. *ELife*, 8. <https://doi.org/10.7554/eLife.46464>
- Killick, R., Fearnhead, P., & Eckley, I. A. (2011). Optimal detection of changepoints with a linear computational cost. *Journal of the American Statistical Association*, 107(500), 1590–1598. <https://doi.org/10.1080/01621459.2012.737745>
- Li, C., Sugam, J. A., Lowery-Gionta, E. G., McElligott, Z. A., McCall, N. M., Lopez, A. J., McKlveen, J. M., Pleil, K. E., & Kash, T. L. (2016). Mu Opioid Receptor Modulation of Dopamine Neurons in the Periaqueductal Gray/Dorsal Raphe: A Role in Regulation of Pain. *Neuropsychopharmacology* 2016 41:8, 41(8), 2122–2132. <https://doi.org/10.1038/npp.2016.12>
- Li, S., Shi, Y., & Kirouac, G. J. (2014). The hypothalamus and periaqueductal gray are the sources of dopamine fibers in the paraventricular nucleus of the thalamus in the rat. *Frontiers in Neuroanatomy*, 0, 136. <https://doi.org/10.3389/FNANA.2014.00136>
- Lin, R., Liang, J., Wang, R., Yan, T., Zhou, Y., Liu, Y., Feng, Q., Sun, F., Li, Y., Li, A., Gong, H., & Luo, M. (2020). The Raphe Dopamine System Controls the Expression of Incentive Memory. *Neuron*, 106(3), 498–514.e8. <https://doi.org/10.1016/j.neuron.2020.02.009>
- Liu, Z., Zhou, J., Li, Y., Hu, F., Lu, Y., Ma, M., Feng, Q., Zhang, J. en, Wang, D., Zeng, J., Bao, J., Kim, J. Y., Chen, Z. F., ElMestikawy, S., & Luo, M. (2014). Dorsal raphe neurons signal reward through 5-HT and glutamate. *Neuron*, 81(6), 1360–1374. <https://doi.org/10.1016/j.neuron.2014.02.010>

- Miyazaki, K. W., Miyazaki, K., Tanaka, K. F., Yamanaka, A., Takahashi, A., Tabuchi, S., & Doya, K. (2014). Optogenetic activation of dorsal raphe serotonin neurons enhances patience for future rewards. *Current Biology*, 24(17), 2033–2040. <https://doi.org/10.1016/j.cub.2014.07.041>
- Muzerelle, A., Scotto-Lomassese, S., Bernard, J. F., Soiza-Reilly, M., & Gaspar, P. (2016). Conditional anterograde tracing reveals distinct targeting of individual serotonin cell groups (B5–B9) to the forebrain and brainstem. *Brain Structure and Function*, 221(1), 535–561. <https://doi.org/10.1007/S00429-014-0924-4/TABLES/3>
- Okaty, B. W., Commons, K. G., & Dymecki, S. M. (2019). Embracing diversity in the 5-HT neuronal system. *Nature Reviews Neuroscience* 2019 20:7, 20(7), 397–424. <https://doi.org/10.1038/s41583-019-0151-3>
- Ozawa, T., Ycu, E. A., Kumar, A., Yeh, L. F., Ahmed, T., Koivumaa, J., & Johansen, J. P. (2016). A feedback neural circuit for calibrating aversive memory strength. *Nature Neuroscience* 2016 20:1, 20(1), 90–97. <https://doi.org/10.1038/nn.4439>
- Paquelet, G. E., Carrion, K., Lacefield, C. O., Zhou, P., Hen, R., & Miller, B. R. (2022). Single-cell activity and network properties of dorsal raphe nucleus serotonin neurons during emotionally salient behaviors. *Neuron*, 110(16), 2664–2679.e8. <https://doi.org/10.1016/j.neuron.2022.05.015>
- Pinault, D. (1994). NEUROSCIENC[IETTERS Golgi-like labeling of a single neuron recorded extracellularly. In *Neuroscience Letters* (Vol. 170).
- Poulin, J. F., Caronia, G., Hofer, C., Cui, Q., Helm, B., Ramakrishnan, C., Chan, C. S., Dombeck, D. A., Deisseroth, K., & Awatramani, R. (2018). Mapping projections of molecularly defined dopamine neuron subtypes using intersectional genetic approaches. *Nature Neuroscience*, 21(9), 1260–1271. <https://doi.org/10.1038/s41593-018-0203-4>
- Ren, J., Friedmann, D., Xiong, J., Liu, C. D., Ferguson, B. R., Weerakkody, T., DeLoach, K. E., Ran, C., Pun, A., Sun, Y., Weissbourd, B., Neve, R. L., Huguenard, J., Horowitz, M. A., & Luo, L. (2018). Anatomically Defined and Functionally Distinct Dorsal Raphe Serotonin Sub-systems. *Cell*, 175(2), 472–487.e20. <https://doi.org/10.1016/j.cell.2018.07.043>
- Roy, M., Shohamy, D., Daw, N., Jepma, M., Wimmer, G. E., & Wager, T. D. (2014). Representation of aversive prediction errors in the human periaqueductal gray. *Nature Neuroscience* 2014 17:11, 17(11), 1607–1612. <https://doi.org/10.1038/nn.3832>
- Schweimer, J. v., & Ungless, M. A. (2010). Phasic responses in dorsal raphe serotonin neurons to noxious stimuli. *Neuroscience*, 171(4), 1209–1215. <https://doi.org/10.1016/J.NEUROSCIENCE.2010.09.058>
- Sengupta, A., & Holmes, A. (2019). A Discrete Dorsal Raphe to Basal Amygdala 5-HT Circuit Calibrates Aversive Memory. *Neuron*, 103(3), 489–505.e7. <https://doi.org/10.1016/J.NEURON.2019.05.029>
- Seo, C., Guru, A., Jin, M., Ito, B., Sleezer, B. J., Ho, Y. Y., Wang, E., Boada, C., Krupa, N. A., Kullakanda, D. S., Shen, C. X., & Warden, M. R. (2019). Intense threat switches dorsal raphe serotonin neurons to a paradoxical operational mode. *Science*, 363(6426), 539–542. https://doi.org/10.1126/SCIENCE.AAU8722/SUPPL_FILE/AAU8722_SEO_SM.PDF
- Steinbusch, H. W. M. (1981). Distribution of serotonin-immunoreactivity in the central nervous system of the rat—Cell bodies and terminals. *Neuroscience*, 6(4), 557–618. [https://doi.org/10.1016/0306-4522\(81\)90146-9](https://doi.org/10.1016/0306-4522(81)90146-9)

- Taylor, N. E., Pei, J., Zhang, J., Vlasov, K. Y., Davis, T., Taylor, E., Weng, F.-J., van Dort, C. J., Solt, K., & Brown, E. N. (2019). The Role of Glutamatergic and Dopaminergic Neurons in the Periaqueductal Gray/Dorsal Raphe: Separating Analgesia and Anxiety. *ENeuro*, 6(1), ENEURO.0018-18.2019. <https://doi.org/10.1523/ENeuro.0018-18.2019>
- Weissbourd, B., Ren, J., DeLoach, K. E., Guenther, C. J., Miyamichi, K., & Luo, L. (2014). Presynaptic Partners of Dorsal Raphe Serotonergic and GABAergic Neurons. *Neuron*, 83(3), 645–662. <https://doi.org/10.1016/J.NEURON.2014.06.024>
- Yu, W., Pati, D., Pina, M. M., Schmidt, K. T., Boyt, K. M., Hunker, A. C., Zweifel, L. S., McElligott, Z. A., & Kash, T. L. (2021). Periaqueductal gray/dorsal raphe dopamine neurons contribute to sex differences in pain-related behaviors. *Neuron*, 109(8), 1365-1380.e5. <https://doi.org/10.1016/J.NEURON.2021.03.001>
- Zhao, Q., Ito, T., Soko, C., Hori, Y., Furuyama, T., Hioki, H., Konno, K., Yamasaki, M., Watanabe, M., Ohtsuka, S., Ono, M., Kato, N., & Yamamoto, R. (2022). Histochemical Characterization of the Dorsal Raphe-Periaqueductal Grey Dopamine Transporter Neurons Projecting to the Extended Amygdala. *ENeuro*, 9(3). <https://doi.org/10.1523/ENeuro.0121-22.2022>