Uncovering the Role of Glycinergic Neurons in the Periaqueductal Grey

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A thesis submitted to fulfil the requirements of the degree of Doctor of Philosophy

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Statement of Originality

I, *Caitlin Emily Fenech,* certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any other degree or purpose.

I certify that the intellectual content of this thesis is the product of my own work, and that any assistance received in preparing this thesis and sources have been acknowledged. Any contributions from fellow lab members, collaborators or my supervisory team have been appropriately credited in each chapter preface.

Caitlin Emily Fenech, 30 September 2025

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Authorship Attribution Statement

Chapter 1 (General Introduction) includes sections and subsections that are adapted from the introduction of my Honours thesis (submitted to the University of Sydney on the 24th of November 2021). This is indicated next to the section heading*.

One peer-reviewed journal article has been included as **Chapter 2** and was published in the Journal of Neurochemistry. I performed research, analysed data and was involved with the research design, writing and appraisal of the manuscript.

> Fenech, C., Winters, B. L., Otsu, Y., & Aubrey, K. R. (2024). Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature. *Journal of Neurochemistry*, 168, 3663–3684. **https://doi.org/10.1111/jnc.16191**

Chapter 3 and 4 is prepared for submission.

Appendix 1 contains a peer-reviewed journal article and was published in eNeuro. I was involved with the research and analysis of the data.

> Assareh, N., Fenech, C., Power, R., Uddin, M. N., Otsu, Y., & Aubrey, K. R. (2023). Bidirectional Modulation of Nociception by GlyT2+ Neurons in the Ventrolateral Periaqueductal Gray. *eNeuro*, 10(6). **https://doi.org/10.1523/ENEURO.0069-23.2023**

I am not the corresponding author of the published articles, and as such permission to include the published material has been granted by the corresponding author.

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As supervisor for the candidature upon with this thesis is based, I can confirm that the authorship attribution statements above are correct.

Lead supervisor: Associate Professor Karin Renee Aubrey, 30 September 2025

Generative AI Attribution Statement

No content produced by generative AI tools has been used in the preparation of this thesis.

Publications, Presentations and Awards

Publications:

Assareh, N., Fenech, C., Power, R, Uddin, M. N., Otsu, Y., Aubrey, K. R. (2023). Bidirectional modulation of nociception by GlyT2+ neurons in the ventrolateral periaqueductal gray. *eNeuro*, 10 (6). **https://doi.org/10.1523/ENEURO.0069-23.2023**

Fenech, C., Winters, B. L., Otsu, Y., & Aubrey, K. R. (2024). Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature. *Journal of Neurochemistry*, 168, 3663–3684. **https://doi.org/10.1111/jnc.16191**

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Fenech, C., Assareh, N., Aubrey, K. R. A standardised look at the Complete Freund's Adjuvant (CFA) model in male and female mice regarding nociception and anxiety-like behaviours. *Manuscript in preparation.*

Oral presentations:

- **2023 Australian Pain Society 43rd Annual Scientific Meeting** - *2-5 April 2023, National Convention Centre, Canberra ACT*
    - **"**Can modulation of glycinergic periaqueductal grey neurons be therapeutic in a chronic pain state?"
    - Fenech C., Assareh N., Aubrey K.; Oral presentation and poster presented by C Fenech
- **Royal North Shore Hospital Scientific Staff Council Annual Research Forum 2023** – *14 December 2023, Kolling Institute of Medical Science, St Leonards NSW*
    - "Uncovering the role of a subpopulation of neurons in pain signalling"
    - Fenech C., Assareh N., Aubrey K.; Oral presentation presented by C Fenech
- **Inaugural Kolling HDR and ECR Research Forum, 2024** – 6 May 2024, Kolling Institute of Medical Science, St Leonards NSW
    - "Uncovering the role of GlyT2+ periaqueductal grey neurons in pain and pain-related behaviours"

- Fenech C., Assareh N., Aubrey K.; Oral presentation presented by C Fenech

- **Kioloa Neuroscience Colloquium 2024 –** 17-19 May 2024, Australian National University Kioloa Coastal Campus, Kioloa NSW
  - **"**Uncovering the role of GlyT2+ periaqueductal grey neurons in pain and pain-related behaviours"
  - Fenech C., Assareh N., Aubrey K.; Oral presentation presented by C Fenech
- **Sydney Pain Consortium 2024** – 3 September 2024, The Sibyl Centre, Women's College, Camperdown NSW
  - "Uncovering the role of a subpopulation of neurons in pain and pain-related behaviours"
  - Fenech C., Assareh N., Aubrey K.; Oral presentation presented by C Fenech
- **4th Optogenetics Meeting Australia** – 6-7 February 2025, Kolling Institute of Medical Science, St Leonards NSW
  - "Uncovering the role and connectivity of glycinergic neurons in the periaqueductal grey"
  - Fenech C., Assareh N., Aubrey K.; Oral presentation presented by C Fenech
- **36th World Congress of Neuropsychopharmacology** – 17- 18 June 2025, Melbourne Convention and Exhibition Centre, VIC
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  - Fenech C., Power R., Otsu Y., Assareh N., Aubrey K.; Oral presentation and poster presented by C Fenech

Poster presentations:

- **Biological Psychiatry Australia 13th Annual Scientific Meeting**- *25-25 October 2023, Pullman Palm Cove Resort, Cairns, QLD*
  - "Is inhibition of GlyT2+ periaqueductal grey neurons therapeutic in a persistent inflammatory pain state?"
  - Fenech C., Assareh N., Aubrey K.; Poster presented by C Fenech
- **Society of Neuroscience 2024 –** 5-9 October 2024, McCormick Place Convention Center, Chicago, United States of America
  - "Uncovering the role of GlyT2+ periaqueductal grey neurons in pain and pain-related behaviours"
  - Fenech C., Assareh N., Aubrey K.; Poster presented by C Fenech

- **Biological Psychiatry Australia 14th Annual Scientific Meeting** – *4-5 November 2024, Mercure Sydney, Sydney NSW*
    - "Uncovering the role of GlyT2+ periaqueductal grey neurons in pain and pain-related behaviours"
    - Fenech C., Assareh N., Aubrey K.; Poster will be presented by C Fenech

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- **Inaugural Kolling HDR and ECR Research Forum, 2024, Outstanding HDR Presentation** – 6 May 2024, Kolling Institute of Medical Science
    - "Uncovering the roles of GlyT2+ periaqueductal grey neurons in pain and pain-related behaviours"
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List of Abbreviations

AAV	Adeno-associated virus
AUC	Area under the curve
CFA	Complete freund's adjuvant
CNS	Central nervous system
CNO	Clozapine N-oxide
CPA	Conditioned place aversion
DR	Dorsal raphe
DREADD	Designer receptors exclusively activated by designer drugs
EGFP	Enhanced green fluorescent protein
eGlyR	Excitatory glycine receptor
EPM	Elevated plus maze
GABA	Gamma aminobutyric acid
GAD65/67	Glutamate decarboxylase 65/67
GFP	Green fluorescent protein
GlyR	Glycine receptor
GLYRA3	Glycine receptor α3 subunit
GlyT2	Glycine transporter 2
ISH	In situ hybridisation
i.p.	Intraperitoneal (injection)
LC	Locus coeruleus
LDT	Light-dark test
LH	Lateral hypothalamus
lPBN	Lateral parabrachial nucleus
OFT	Open field
PAG	Periaqueductal grey
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PVT	Paraventricular thalamus
ROI	Region of interest
REM	Rapid eye movement sleep
RT	Room temperature
RVM	Rostral ventromedial medulla
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
VIAAT	Vesicular inhibitory amino acid transporter
vGAT	Vesicular GABA transporter
vlPAG	Ventrolateral PAG
VTA	Ventral tegmental area

Abstract

Chronic pain is a debilitating health condition. In Australia alone it affects almost 4 million people and has a burden of 20% of the world's population. Chronic pain is a multifaceted biopsychosocial condition that can have an extensive effect on a person's quality of life and is often linked with comorbidities such as depression, anxiety and sleep disorders. Therefore, there is a crucial need for an improved understanding of the brain circuits that contribute to pain signalling and their role in the development of chronic pain. The midbrain periaqueductal grey (PAG) is an established integrator of pain and related behaviours and consists of over 100 different neuronal subpopulations. The inhibitory neurotransmitter glycine plays a significant role in pain signalling in the spinal cord and a population of glycinergic neurons are distributed in the ventrolateral column of the PAG (PAG^{GlyT2}). PAG^{GlyT2} neurons have been previously demonstrated to bidirectionally modulate nociception in a naïve state. The characterisation and role of these neurons in other PAG mediated behaviours was unknown as well as their role in chronic pain states. Therefore, the overarching aim of this thesis was to further characterise the role of glycinergic neurons in the PAG in both naïve and chronic pain states.

Chapter 2 examined the current evidence supporting a role for glycine signalling in supraspinal pain modulation in the form of a scoping review. I found clear evidence that glycinergic neurotransmission plays a role in nociception in brain circuitry and is implicated in a wide range of other behaviours. **Chapter 3** aimed to further characterise the distribution, projections and role of PAG^{GlyT2} neurons in a naïve state, through circuit tracing, electrophysiology and behavioural chemogenetic experiments. We found that PAG^{GlyT2} neurons are a subset of inhibitory projection neurons in the vlPAG that sense and modulate nociception and affective behaviours such as anxiety and aversion. I next wanted to investigate the role of PAG^{GlyT2} neurons in a chronic pain state, so systematically characterised nociception and anxiety-like behaviours in the CFA-induced inflammatory persistent pain preclinical model in **Chapter 4.** This guided the experimental design for **Chapter 5** where I investigated the role of PAG^{GlyT2} neurons in the CFA-induced inflammatory persistent pain model using chemogenetics. I discovered that the role of PAG^{GlyT2} neurons shifts between a naïve and chronic pain state (and between sex) and neuronal activity marking suggests that engagement of different projection regions may contribute to this. Overall, this thesis uncovers the complex role of PAG^{GlyT2} neurons and reveals novel midbrain circuitry for modulating nociception and affective behaviours in naïve and chronic pain states in both males and females.

Thesis Outline

Chapter 1: General Introduction

Introduces the overall thesis and justification for this project.

Chapter 2: Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature

Gives a broad overview of the role of supraspinal glycinergic neurotransmission in pain, with a subsection on what is known about its role in the periaqueductal grey.

Chapter 3: Glycinergic neurons in the periaqueductal grey are a novel population of inhibitory projection neurons involved in pain, anxiety and aversion

Extends the current knowledge on the properties and role of glycinergic neurons in the periaqueductal grey. However, their role in chronic pain conditions is unknown.

Chapter 4: A standardised look at the Complete Freund's Adjuvant (CFA) model in male and female mice regarding nociception and anxiety-like behaviours

Defines nociception and anxiety-like behaviours in a commonly used chronic pain preclinical model, known as the Complete Freund's Adjuvant (CFA) induced persistent inflammatory pain model to guide experimental design for Chapter 5.

Chapter 5: The role of glycinergic neurons in the periaqueductal grey is altered in a chronic inflammatory pain state

Builds on the findings in Chapter 3 and 4 and investigates the role of glycinergic neurons in the periaqueductal grey in a chronic pain preclinical model.

Chapter 6: General Discussion

Provides a general discussion on the findings and implications of the thesis.

Chapter 1: General Introduction

1.1 Pain is a complex experience

The International Association for the Study of Pain defines pain as "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage". This definition encapsulates that pain is not solely defined through nociception and that it is a personal complex experience (Raja et al., 2020). Pain is important as an evolutionary tool to prevent or minimise tissue damage in response to environmental dangers and then to avoid them in the future. Thus, it is both a sensory and affective experience, often causing much distress and unpleasantness (Price, 2000). In some cases, this acute protective pain experience shifts into a maladaptive, harmful chronic pain state.

1.1.1 *Chronic pain is a significant health issue

Chronic pain is usually defined as pain that continues for more than three months, persisting past normal healing time (Bonica & Hoffman, 1954; Treede et al., 2019). In Australia, it affects almost 4 million people and in a 2024 report, it was revealed that almost half of those living with chronic pain are of working age, resulting in large impacts on national productivity (Blyth et al., 2001; Chronic Pain Australia, 2024). Globally, it is estimated that chronic pain has a burden of 20% of the world's population (Treede et al., 2015). Chronic pain is a multifaceted biopsychosocial condition that can have an extensive effect on a person's life, affecting their ability to work, sleep, complete cognitive tasks and their overall quality of life (Finan et al., 2013; Fine, 2011). Additionally, chronic pain is often linked with mental health conditions such as depression, anxiety, and sleep disorders (Treede et al., 2019). Many long-term health conditions such as arthritis, cancer, endometriosis, and migraines are associated with an increased risk of chronic pain (Dominick et al., 2012).

Due to its complex nature, current pharmacotherapies (i.e., opioids, gabapentanoids, and antidepressants) are not helpful for many patients. In chronic neuropathic pain, clinically meaningful pain relief is only achieved in less than half of patients (Dworkin et al., 2010). Furthermore, opioids are linked with several harmful side effects and carry a risk of dependence and accidental overdose (Kosten & George, 2002). To develop more effective drug therapies and provide better patient care, there is a crucial need for an improved understanding of the brain circuits that contribute to pain signalling and their role in the development of chronic pain.

1.2 The periaqueductal grey is an integrative hub of pain and related behaviours

The midbrain periaqueductal grey (PAG) is an essential part of the neural circuitry that helps coordinate appropriate responses to various stressors, including pain. It achieves this through extensive efferent and afferent connections throughout the brain including to the medulla, pons, thalamus and hypothalamus (Cameron, Khan, Westlund, Cliffer, et al., 1995; Cameron, Khan, Westlund, & Willis, 1995; K. A. Keay & Bandler, 2015). The PAG is characteristically divided into functional columns, with the ventrolateral column particularly important for opioid-mediated analgesia through the descending pain pathway (Lau & Vaughan, 2014). In addition, it is made up of many different neuronal subpopulations that further contribute to its diverse control of behaviours (Vaughn et al., 2022; H. Zhang et al., 2024).

1.2.1 *The PAG is divided into functional columns

The PAG can be anatomically separated into neuronal columns, with different afferents, efferents and overall function. The four columns are the dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG) and the ventrolateral (vlPAG) subdivisions (K. Keay & Bandler, 2008).

The dmPAG is often linked with the neuromodulator serotonin, and activation of these serotonergic dmPAG neurons has been shown to be antinociceptive (Baptista-de-Souza et al., 2020; de Freitas et al., 2014). In addition, inputs from the ventromedial hypothalamus, have been linked with panic-like behaviours in rats (Ullah et al., 2017).

The other PAG columns have been implicated in modulating different emotional coping responses to stress, see Figure 1 (K. Keay & Bandler, 2008). Pharmacological activation, via excitatory amino acids, of the dlPAG and lPAG columns produces an 'active emotional coping' response. This is characterised by 'fight-or-flight' behaviours in response to a threat and associated with tachycardia and hypertension. Activation of the rostral side of these columns evokes a confrontational defensive response ('fight'), whereas an escape response ('flight') is linked with activation of the caudal side of the dlPAG and lPAG. These behaviours are often evoked if the stressor is escapable and controllable. In contrast, pharmacological activation of the ventrolateral PAG (vlPAG) column produces a 'passive emotional coping' response, which is characterised by quiescence and hyporeactivity. As such, the vlPAG is activated in response

to an inescapable stressor (e.g., persistent pain) and associated with hypotension and bradycardia (Bandler et al., 1985; Bandler & Carrive, 1988; K. A. Keay & Bandler, 2015).

Importantly, low doses of morphine (an opioid) produce antinociception exclusively when injected in the vlPAG region of the PAG, and thus, the vlPAG is often a target area to investigate pain regulation (Bobeck et al., 2009).

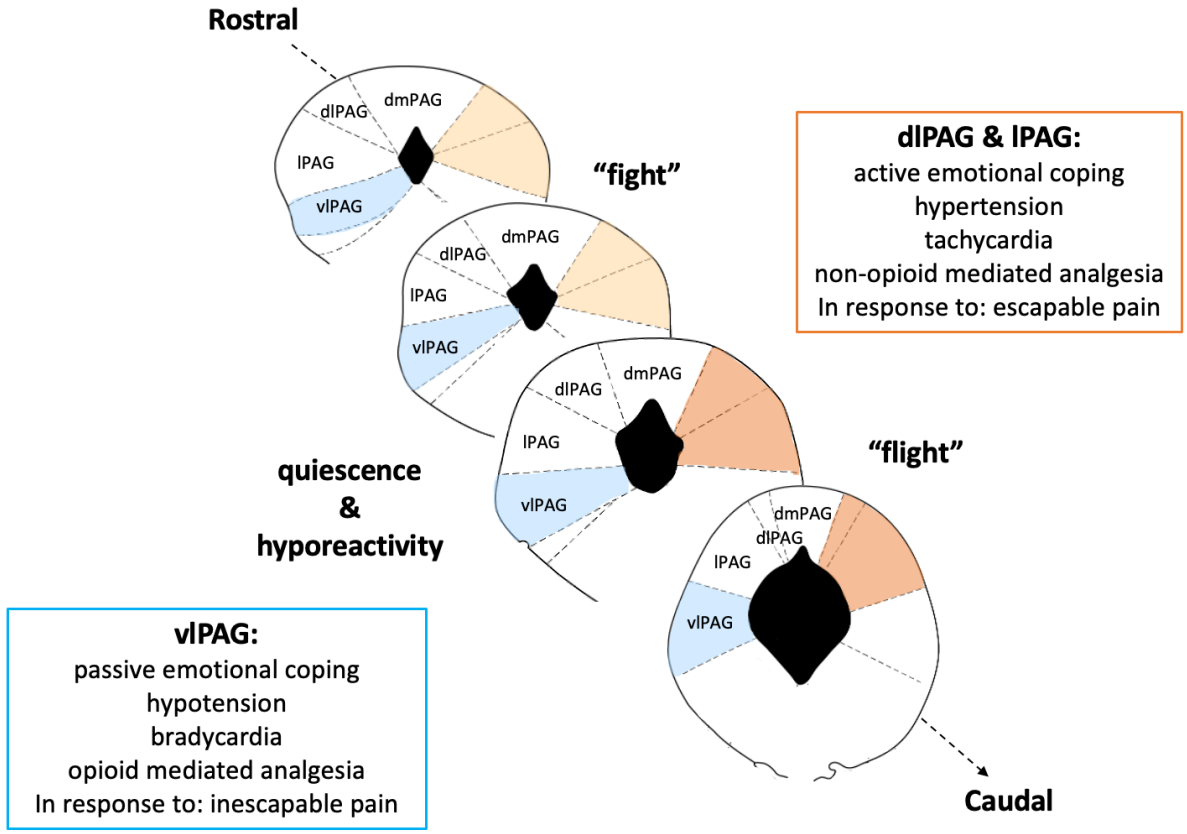

Figure 1: Schematic illustration of the midbrain periaqueductal grey neural columns. Injections of excitatory amino acids within the dorsolateral (dlPAG) and lateral (lPAG) PAG, evoke 'active emotional coping' strategies, which involves 'fight-or-flight' behaviours, hypertension, tachycardia, and non-opioid mediated analgesia. In contrast, injections of excitatory amino acids within the ventrolateral PAG (vlPAG), evoke 'passive emotional coping' strategies, which involves quiescence and hyporeactivity, hypotension, bradycardia, and opioid-mediated analgesia. The dlPAG and lPAG are activated in response to escapable pain, whereas the vlPAG is activated in response to inescapable pain. Adapted from Bandler, R & Shipley, MT 1994, 'Columnar organization in the midbrain periaqueductal gray: modules for emotional expression?', Trends Neurosci, vol. 17, no. 9, pp. 379-89.

1.2.2 *The PAG involvement with the descending pain pathway

The descending pain pathway acts as an endogenous analgesic system through the central nervous system (CNS). A major descending pathway originates in the vlPAG and projects to the spinal dorsal horn via the rostral ventromedial medulla (RVM; see Figure 2). At the level of the spinal cord, it directly modulates the incoming nociceptive transmission and thus, evokes analgesia (Fields & Basbaum, 1978; Lau & Vaughan, 2014). Electrical stimulation of the PAG elicits an analgesic response and this is achieved through inhibiting the nociceptive signal in the spinal cord (Behbehani, 1995). When chronic pain develops, there is a dysregulation in the descending system, which contributes to a loss of this inhibition and spinal cord sensitization (De Felice et al., 2011; Millan, 2002).

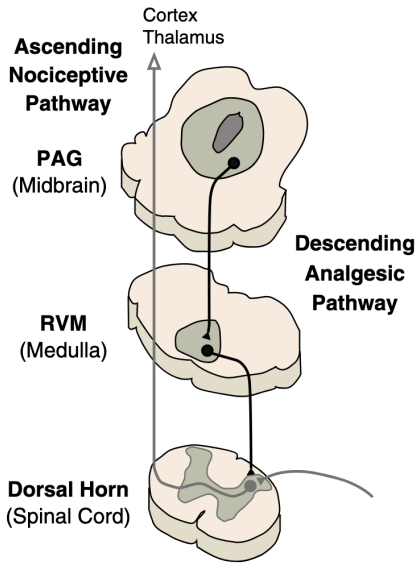

Figure 2: Schematic of an ascending pain pathway (left) and descending analgesic pathway (right). This descending pain pathway originates in the PAG and projects to the spinal cord via the RVM to modulate the incoming noxious transmission at the level of the spinal cord. From Lau, BK & Vaughan, CW 2014, 'Descending modulation of pain: the GABA disinhibition hypothesis of analgesia', Current Opinion in Neurobiology, vol. 29, pp. 159-164.

The descending pain pathway is mediated through cannabinoid and opioid signalling (Vaughan et al., 1997; Winters et al., 2022). Classically, tonically active inhibitory GABAergic interneurons in the PAG are suppressed by opioids and cannabinoids allowing for the activation of excitatory glutamatergic vlPAG projection neurons to the RVM, a process known as disinhibition. This in turn activates RVM projection neurons to the dorsal horn of the spinal cord allowing for analgesia (Depaulis et al., 1987; Lau & Vaughan, 2014; Moreau & Fields,

1986; Vaughan et al., 1997). However, recent developments in neuroscience techniques have allowed for the study of different subpopulations of neurons in the PAG and have expanded what is known about PAG circuitry and function.

1.2.3 There are many different neuronal subtypes in the PAG

Optogenetics and chemogenetics have allowed for the selective modulation of subpopulation of neurons and selective modulation of inputs and/or outputs (Deisseroth, 2011; Roth, 2016). Further, fibre photometry studies have allowed for the *in vivo* investigation of the activity of a subpopulation of neurons or even the activity of specific neurotransmitters or neuropeptides (T.-W. Chen et al., 2013; Sun et al., 2020). A recent study using spatial transcriptomics has identified 144 different neuronal subpopulations in the PAG, expanding the computational ability of this one region (Vaughn et al., 2022). Using this same technique, another study further classified 41 neuronal subpopulations based on their pain-encoding roles (Kimmey et al., 2025).

The vlPAG is mainly known for its role in modulating pain and analgesia, but these techniques have allowed for the understanding about this region to be expanded in great detail. In addition to defensive responses, the ventrolateral columns of the PAG have also been implicated in pain, fear, predatory hunting, anxiety, depression, cardiovascular function, respiratory regulation, sleep, and bladder control (see Zhang et al., 2024 for a comprehensive review). The current knowledge about the role of subpopulation of neurons in the vlPAG, as well as the regions inputs and outputs, is summarised below in Table 1 and 2:

Table 1: The neural circuits and functions of vlPAG inputs. Adapted from Zhang et al., 2024.

Function	Region/Neural Circuit	Neuronal subpopulation		Related behaviours	Reference
		vlPAG input	vlPAG		
Pain regulation	PVN-vlPAG	OT	OTR+	Analgesic in both inflammatory and neuropathic pain models	(Iwasaki et al., 2023)
	LH-vlPAG	PV	Glu	Pain behaviours	(Siemian et al., 2021)
	RE-mPFC-vlPAG	Glu; GABA	Glu	Pain and depression-like behaviours in neuropathic pain	(Bao et al., 2025)
	PrL-l/vlPAG	Glu	Glu	Chronic pain-related hyperalgesia	(F. Gao et al., 2023)
	vlOFC-vlPAG	Glu	\	Hypersensitivity after peripheral nerve injury	(J. Huang et al., 2021)
	vlOFC-vlPAG	Glu	\	Trigeminal neuropathic pain	(Islam et al., 2025)
	PVP-Zir-vlPAG	Glu; GABA	Glu	Facilitation of neuropathic pain	(D. Li et al., 2024)
	pPVT-CeA-vlPAG	Glu; \	Glu	Neuropathic pain	(Liang et al., 2020)
	mPFC-vlPAG	MOR+ /GABA	\	Placebo analgesia	(Neyama et al., 2025)
	VTA-mPFC-vlPAG	DA; Glu	\	Neuropathic pain associated behaviours	(S. Huang et al., 2020)
	rACC-vlPAG	Glu	\	Electroacupuncture	(X. Zhu et al., 2022)
	vLGN/IGL-l/vlPAG	GABA	GABA	Antinociceptive effects of bright light treatment	(Hu et al., 2022)
	BLA-mPFC-vlPAG	Glu; \	\	Nociception, pain affect and cognition	(J. Huang et al., 2019)
Defensive behaviour	CeA-vlPAG	GABA	Glu/GABA	Conditioned freezing	(Tovote et al., 2016)
	AHN-vlPAG	GABA	\	Defensive attacks and biting	(Z. Xie et al., 2022)
	mCbN-vlPAG	Glu	DA	Freezing behaviours	(Vaaga et al., 2020)
	FN-vlPAG	Glu	Glu/GABA	Fear conditioning	(Frontera et al., 2020)
	MCN-vlPAG	\	\	Fear learning	(Lawrenson et al., 2021)
	mPFC-vlPAG	\	GABA	Stress-coping response	(Skog et al., 2024)
	dmPFC-l/vlPAG	\	\	Fear discrimination	(Rozeske et al., 2018)
Predatory hunting	LH-l/vlPAG	GABA	\	Evasion; Predatory hunting	(Rossier et al., 2021)
	MPOA-LH-vlPAG	Glu; Glu	\	Predatory eating in hunting behaviour	(Tan et al., 2022)
	ZI-PAG	GABA	\	Hunting	(Zhao et al., 2019)
	BF-PAG	GABA	\	Hunting and instrumental responding and consumption for food	(Roman-Ortiz et al., 2021)
	LH/BNST-vlPAG	GABA	GABA	Feeding behaviour	(Hao et al., 2019)
Anxiety and depression	dmPFC-vlPAG	Glu/GABA	Glu	Pain threshold maintenance and anti-anxiety behaviours	(Yin et al., 2020)
	MPOA-PAG	Glu	\	Anxiety-like behaviours	(G.-W. Zhang et al., 2021)
Sleep/wake	VM/LH-vlPAG	GABA/ MCH	\	Promotion of REM sleep	(Kroeger et al., 2019; Weber et al., 2015)

	mPFC-vlPAG-VTA	Glu	GABA	Emergence from anaesthesia	(Guo et al., 2023)
Others	LPB-l/vlPAG	Glu	\	Modulation in itching sensation	(J.-N. Li et al., 2021)
	BNST-vlPAG	5-HT	\	Operant alcohol self-administration	(Flanigan et al., 2024)
	PrL-vlPAG	\	\	Adolescent alcohol exposure	(Obray et al., 2025)

Table 2: The neural circuits and functions of vlPAG neuronal subpopulations and outputs.

Adapted from Zhang et al., 2024.

Function	**Region/Neural Circuit**	**Neuronal subpopulation**		**Related behaviours**	**Reference**
		vlPAG	**vlPAG output**		
Pain regulation	vlPAG/DR	GABA	\	Nociception	(L. Xie et al., 2023)
	vlPAG/DR-BNST	DA	\	Nociception and locomotion	(Yu et al., 2021)
	vlPAG	Glycine	\	Nociception and locomotion	(Assareh et al., 2023)
	vlPAG-DR	\	5-HT	Preoperative chronic pain with anaesthesia induces postoperative cognitive dysfunction	(Deng et al., 2023)
	vlPAG	Glu	\	Trigeminal neuralgia	(Elina et al., 2021)
	vlPAG	CB1R+	\	Analgesia	(Jiang et al., 2022)
	vlPAG	MOR+	\	Analgesia	(Kimmey et al., 2025)
	vlPAG-VTA/RVM	Glu	\	Chronic pain induced depression-like behaviour	(Lee et al., 2023)
	vlPAG/DR	DA	\	Regulation of pain	(C. Li et al., 2016)
	vlPAG/DR	DA	\	Pain and depression comorbidity	(Liu et al., 2023)
	vlPAG	Glu/GABA	\	Nociception and itch	(Samineni et al., 2017, 2019)
	vlPAG-dLS	Glu	\	Acute restraint stress and pain modulation	(Shah et al., 2025)
	vlPAG/DR	DA/Glu	\	Anxiety and analgesia	(Taylor et al., 2019)
	vlPAG-VTA	Glu/GABA	\	Headache aversiveness	(Waung et al., 2019)
	vlPAG	\	\	Diabetic neuropathic pain	(Yang et al., 2022)
	l/vlPAG-RVM	SST	\	Chemotherapy-induced neuropathic pain	(Y. Zhang et al., 2023)
	vlPAG	GABA/CB1R+	\	Electroacupuncture	(H. Zhu et al., 2019)
	vlPAG	Glu	\	Electroacupuncture	(X. Zhu et al., 2024)
Defensive behaviour	vlPAG-Mc	Glu	\	Freezing behaviours	(Tovote et al., 2016)
	vlPAG-VTA	GABA	\	Freezing behaviours	(Laurent et al., 2020)
	l/vlPAG-LH	Glu/GABA	\	Evasion and unconditioned aversion	(Y. Li et al., 2018)
	PAG	NPY	\	Behavioural resilience to stress exposure in an animal model of post-traumatic stress disorder	(Cohen et al., 2012)

	l/vlPAG-Mc	Glu	\	Defensive responses and behavioural aversion	(Honshuku et al., 2025)
	l/vlPAG	CCK	\	Defensive behaviour	(La-Vu et al., 2022)
	vlPAG-LC	Glu	\	Arousal states in threatening contexts	(S. Wang et al., 2025)
	vlPAG-RVM	SST	\	Pain suppression during defensive states	(Winke et al., 2025)
	vlPAG	5-HT	\	Anxiety-like behaviour induced by acute stress	(C. Yan & Liu, 2024)
Anxiety and depression	vlPAG	GABA	\	Anxiety and conditioned fear	(Lowery-Gionta et al., 2018)
	vlPAG	DOR+	\	Hypotension caused by LPS	(Millington et al., 2016)
Cardio-vascular and respiratory regulation	vlPAG	Glu	\	Cardiovascular regulation during normal and haemorrhagic conditions	(Alikhani et al., 2021)
	l/vlPAG-KFn	\	\	Regulation of upper airway patency	(Trevizan-Baú et al., 2021)
	vlPAG	GABA	\	REM and NREM sleep	(Weber et al., 2018)
Sleep/wake	vlPAG-VTA	GABA	DA	Anaesthesia recovery by nociception	(C.-C. Zhong et al., 2024)
	vlPAG-GiV	NTS	GABA	Non-REM sleep	(P. Zhong et al., 2019)
	vlPAG	DA	\	Arousal	(Porter-Stransky et al., 2019)
	PAG-NTS	GABA	\	Cough-like hypersensitivity	(Z. Chen et al., 2022)
Others	l/vlPAG-RVM-SC	Tac1	\	Itch-Scratching cycle	(Z.-R. Gao et al., 2019)
	vlPAG	YTHDF1	\	Morphine withdrawal	(Ou et al., 2024)
	vlPAG/DR-BNST	DA	\	Alcohol-use disorder	(Pati et al., 2023)
	l/vlPAG-PMC	SST	\	Urination	(Rao et al., 2022; J. Yan et al., 2025)
	l/vlPAG-ZI	GABA	\	Exploration, foraging and hunting	(Reis et al., 2024)
	vlPAG	GABA	\	High-fat diet-induced obesity	(X. Wang et al., 2023)
	vlPAG-BNST	\	\	Feeding regulation	(J. Zhang et al., 2023)

Key for Table 1 and 2: The symbol "/" means to show that the study studied more than one neuronal population/brain region; The symbol ";" means to distinguish between the circuit. i.e. Glu; GABA = Glutamatergic neurons at first region of circuit and GABAergic at second region of circuit; The symbol "\" means the neuronal subpopulation is unknown/output not described. Abbreviations: OT: oxytocin, OTR: oxytocin receptor, PV: parvalbumin, Glu: glutamate, GABA: gamma aminobutyric acid, MOR: μ-opioid receptor, DOR: delta opioid receptor, DA: dopamine, CB1R: cannabinoid r1 receptor, MOR: mu opiod receptor, MCH: melanin concentrating hormone, SST: somatostatin, NPY: neuropeptide-Y, 5-HT: serotonin, CCK: cholecystokinin, NTS: neurotensin. AHN: anterior hypothalamic nucleus, BF: basal forebrain, BLA: basolateral amygdala, BNST: bed nucleus of the stria terminalis, CeA: central amygdala, dLS: dorsal lateral septum, dmPFC: dorsal medial prefrontal cortex, DR: dorsal raphe, FN: cerebellar fastigial nucleus, GiV: gigantocellular reticular nucleus, IGL: intergeniculate leaflet, KFn: Kölliker-Fuse nucleus, LH: lateral hypothalamus, LPB: lateral parabrachial nucleus, Mc: magnocellular nucleus of the medulla, mCbN: medial (fastigial) cerebellar nuclei, MCN: medial cerebellar nucleus, mPFC: medial prefrontal cortex, MPOA: medial preoptic area, PMC: pontine micturition center, pPVT: posterior paraventricular thalamus, PrL: prelimbic cortex, PVN: paraventricular nucleus, PVP: paraventricular thalamic nucleus, rACC: rostral anterior cingulate cortex, RE: nucleus reuniens of the thalamus, vLGN: ventral lateral geniculate nucleus, vlOFC: ventrolateral orbitofrontal cortex, VM: ventral medulla, VTA: ventral tegmental area, VTA: ventral tegmental area, ZI: zona incerta, ZIr: rostral zona incerta.

Therefore, the PAG, and specifically **the vlPAG, is an important part of the pain signalling pathway as well as other behaviours.** One of the neuronal subpopulations in the PAG is defined by the expression of the glycine transporter GlyT2 (Zafra et al., 2016; Zeilhofer et al., 2005). These neurons have a unique restricted distribution in the vlPAG and are likely to be colocalised with GABAergic inhibitory neurons (Rampon et al., 1996; Vaughn et al., 2022; Zeilhofer et al., 2005). This is because glycine and GABA are often colocalised in neurons (Chéry & de Koninck, 1999; Keller et al., 2001) and can be co-released from single vesicles (Jonas et al., 1998). GABA and glycine share the same vesicular transporter (VIAAT or vGAT) that concentrates both GABA and glycine into synaptic vesicles (Wojcik et al., 2006). Co-release of GABA and glycine has been demonstrated at central synapses in the brainstem (Dufour et al., 2010; Nabekura et al., 2004), cerebellum (Dumoulin et al., 2001) and spinal cord (Jonas et al., 1998). The role of GABAergic neurons in the vlPAG (Table 1 and 2) can provide insights into the potential roles of glycinergic neurons in this region and previously we have shown that the activity of GlyT2-positive neurons in the vlPAG are able to bidirectionally modulate nociception (Assareh et al., 2023; see Appendix 1).

1.3 Glycinergic neurons and pain

There are two fast inhibitory neurotransmitters in the CNS: gamma aminobutyric acid (GABA) and glycine. In contrast to GABAergic neurotransmission, there is relatively little known about the inhibitory glycine system, especially its role in the supraspinal regions.

1.3.1 *The inhibitory neurotransmitter glycine plays a critical role in pain signalling in the spinal cord

Glycine is famously involved with motor control but also plays a critical role in pain signalling. These roles have been known since the early 20th century due to the symptoms of strychnine poisoning, a selective competitive glycine receptor antagonist (Yamarick et al., 1992). Poisoning with strychnine causes muscle rigidity and spasm of the facial muscles, ('risus sardonicus'), and without treatment it can lead to asphyxia, cardiac arrest and death (Libenson & Yang, 2001). Additionally in mice, injection of strychnine into the spinal cord causes an increase in tactile sensitivity that resembles allodynia (hypersensitivity to non-noxious stimuli) (Yaksh, 1989).

Glycine's functions can also be illustrated through the rare human disease known as hyperekplexia (also known as hereditary startle disease), which involves a loss of function mutations in genes encoding for glycine receptors and transporters (Andermann et al., 1980). This largely neuromotor disease is characterised by muscle spasms and tremors but interestingly, patients with hyperekplexia also display lower pain thresholds than healthy controls in behavioural tests such as pressure pain detection, electrical stimulation of the ankle and submersion of hands in ice-water (Vuilleumier et al., 2018), underscoring a likely role for glycine signalling in pain perception in humans.

Glycine transporter 2, GlyT2, is essential for glycine uptake into the presynaptic terminal (Aubrey et al., 2007; Gomeza et al., 2003; Rousseau et al., 2008) and thus can be used as a marker for all glycinergic neurons. Mice models that are congenitally deficient in GlyT2, also present fatal neuromotor symptoms that resemble hyperekplexia, because of deficient glycinergic inhibition (Gomeza et al., 2003).

Glycine's role in pain signalling has been most extensively studied in the spinal cord (Peiser-Oliver et al., 2022; Zeilhofer, 2005) where glycine neurons are most highly concentrated. Here, antagonists of glycine receptors produce hypersensitivity to non-noxious stimuli (Yaksh, 1989) and glycinergic neurons have been found to keep non-noxious stimuli from activating noxious pathways in models of neuropathic pain (Lu et al., 2013), indicating important glycine roles in spinal nociceptive processing. Through the use of a transgenic mouse model known as GlyT2::Cre, one study was able to selectively ablate, silence and activate glycinergic neurons in the spinal cord and demonstrate they have a role in both pain and itch (Foster et al., 2015). In addition, the glycine receptor α3 subunit (encoded by the GLYRA3 gene) is selectively distributed in the outer laminae of the spinal cord dorsal horn. GLRA3-knockout mice models have reduced central pain sensitisation (Harvey et al., 2004) and in a model of inflammatory hyperalgesia (enhanced sensitivity to pain) the mice have decreased levels of writhing responses (Rácz et al., 2005). Together, these findings suggest glycine signalling through glycine receptors which contain the α3 subunit are particularly involved in spinal nociception and inflammatory pain.

1.3.2 *Glycinergic neurons have a unique distribution in the brain

As GlyT2 can be used as a marker for all glycinergic neurons, Zeilhofer et al. (2005) developed a bacterial artificial chromosome transgenic mouse model that expressed the enhanced green fluorescent protein (EGFP) under the promoter of GlyT2 and visualised all CNS glycinergic

neurons in the mouse brain (Figure 3, left). The distribution of glycinergic fibres and cell bodies in this model corroborated previous reports in the literature (Rampon et al., 1996; Tanaka & Ezure, 2004; van den Pol & Gorcs, 1988), and allowed for a comprehensive insight into distribution of glycine neurons, as well as facilitated further investigations of glycine's role in the central nervous system (Aubrey & Supplisson, 2018; Dugué et al., 2005; Giber et al., 2015; Rousseau et al., 2008). In contrast to GABAergic neurons (Figure 3, right), glycine neurons are restricted to the spinal cord, cerebellum, and hindbrain, suggesting that these two inhibitory neurotransmitter systems have specialised roles in the brain.

In the hindbrain, high concentrations of glycine cell bodies were found in medullary regions like the spinal trigeminal nucleus, area postrema, parvocellular reticular nucleus, vestibular nucleus, deep cerebellar nuclei, and the rostral ventromedial medulla. At the level of the pons and midbrain, a substantial number of glycinergic cell bodies were found in the superior olive, nucleus of the trapezoid body, pontine reticular nucleus, subcoerulus and the periaqueductal grey. Additionally, dense glycine fibres were found throughout most of the hindbrain (medulla, pons) and some parts of the thalamus and hypothalamus. The hippocampus, amygdala and basal forebrain were found to have light fibre densities, and only occasional glycine fibres were found in the cortex (Rampon et al., 1996; Zeilhofer et al., 2005).

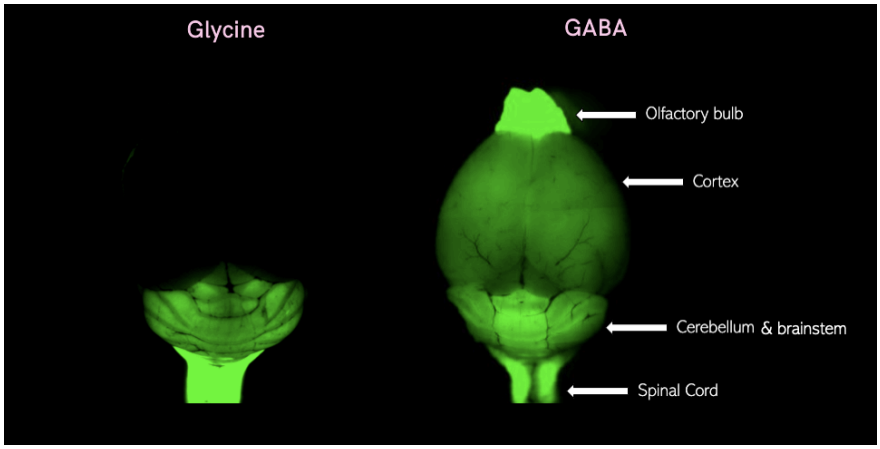

Figure 3: Global distribution of glycinergic (left) and GABAergic neurons (right) in two transgenic mouse models. Enhanced green fluorescent protein (EGFP) is expressed under the promoter of either the glycine transporter GlyT2 (Zeilhofer et al., 2005) or the enzyme GAD67 that makes GABA (Tamamaki et al., 2003) in the mouse brain. Therefore, neurons are labelled green that release these neurotransmitters.

Despite multiple studies reporting that a population of glycinergic cell bodies are found in the PAG (Rampon et al., 1996; Tanaka & Ezure, 2004; van den Pol & Gorcs, 1988; Zeilhofer et al., 2005), and one report suggesting that this population is concentrated in the vlPAG and sends projections to the dorsal raphe, locus coeruleus and the paraventricular nucleus of the hypothalamus (Rampon et al., 1996, 1999; Varga et al., 2019), my laboratory's study was the first to investigate the functional role of glycinergic neurons in the PAG on pain behaviours (Assareh et al., 2023).

1.3.3 Glycinergic neurons in the vlPAG bidirectionally modulate nociception

By utilising a chemogenetic strategy and the genetically modified GlyT2::Cre mouse (Foster et al., 2015), we were able to selectively activate and inhibit the subpopulation of glycinergic neurons in the vlPAG and investigate the effect on nociception and locomotion (See Appendix 1 for full publication).

We first replicated previously reported data and illustrated that GlyT2-positive neurons have a restricted distribution in the ventrolateral column of the PAG of GlyT2::Cre mice (Figure 4).

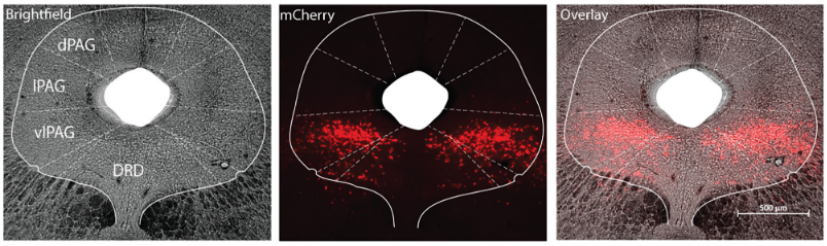

Figure 4: PAG^{GlyT2} neurons have a restricted distribution to the vlPAG. Representative microscopic images of coronal sections showing AAV5-hSyn-DIO-hM4D(Gi)-mCherry stereotaxic injection into the PAG of GlyT2::Cre mice: showing the expression of brightfield (grey), mCherry fluorescence (red) and an overlay of brightfield and mCherry. Scale bars: 500μm, magnification: 2.5X. Bregma AP: -4.72 mm. Figure from Assareh et al., 2023.

We next used DREADDs to demonstrate that the activity of these GlyT2-positive neurons in the PAG (PAG^{GlyT2}) can bidirectionally modulate acute nociceptive responses. We stereotaxically injected Cre-dependent control, excitatory DREADDs (hM3Dq) or inhibitory DREADDs (hM4Di), into the PAG of GlyT2::Cre male mice. 3-4 weeks after injection, to allow for viral transduction, an acetone and hotplate test was conducted on the animals to assess acute nociceptive behaviours, before and after vehicle or CNO i.p. injection. We found that chemogenetic activation of PAG^{GlyT2} neurons, significantly increased responses in the acetone

test and decreased paw withdrawal latency in the hotplate test, indicating an increase in nociceptive sensitivity. While chemogenetic inhibition of PAG^{GlyT2} neurons resulted in the opposite. Therefore, chemogenetic activation of PAG^{GlyT2} neurons can bidirectionally modulate acute thermal nociception (Figure 5).

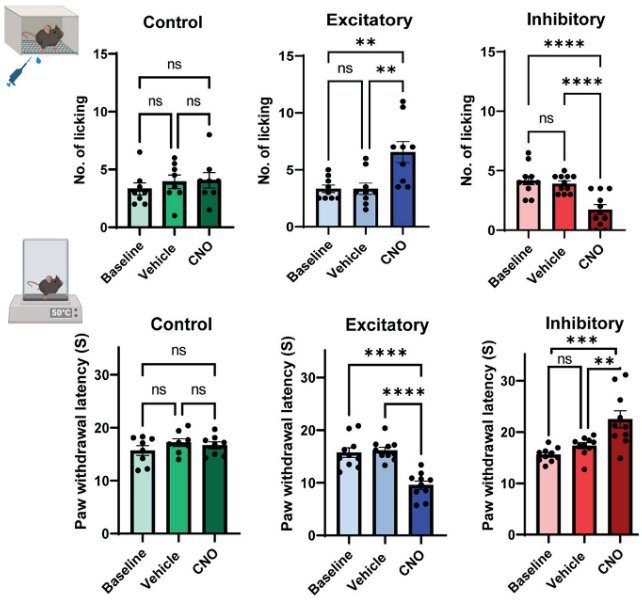

Figure 5: Chemogenetic modulation of PAG^{GlyT2} neurons bidirectionally modulates acute nociceptive behaviour in male GlyT2::Cre mice. CNO (3 mg/kg, i.p.) administration resulted in increased hind paw licking and decreased PWL in GlyT2::cre male animals injected with hM3Dq (blue bars). CNO (5 mg/kg, i.p.) administration resulted in decreased hind paw licking and increased PWL in GlyT2::cre animals injected with hM4Di (red bars) compared with vehicle injection. CNO (5 mg/ kg, i.p.) administration had no effect on hind paw licking and PWL in GlyT2::cre animals injected with control mCherry vector (green bars). Individual animals are indicated on the graphs (n = 7–11) and values are presented as mean 6 SEM, and significant results were determined when *p< 0.05. Figure adapted from Assareh et al., 2023.

Next, we investigated the effect of chemogenetic modulation of PAG^{GlyT2} neurons on locomotor activity. Chemogenetic activation resulted in an increase in locomotion (total distance moved and total time of mobility) as well as an increase in jumping responses in male mice (Figure 6). Although preclinical research has been traditionally carried out using male animals, there is increasing evidence that there are substantial sex differences in pain responses and pathways in animals (Mogil, 2012), including in the PAG (Jiang et al., 2022; Linnman et al., 2012; Loyd et al., 2007; Tonsfeldt et al., 2016; Yu et al., 2021). Thus, in a small cohort of animals, we also investigated chemogenetic modulation of PAG^{GlyT2} neurons in female GlyT2::Cre mice and found that they modulate acute nociceptive behaviours in females as well. Chemogenetic

activation of PAG^{GlyT2} neurons in females altered locomotor activity however it affected the quality of locomotor behaviours differentially.

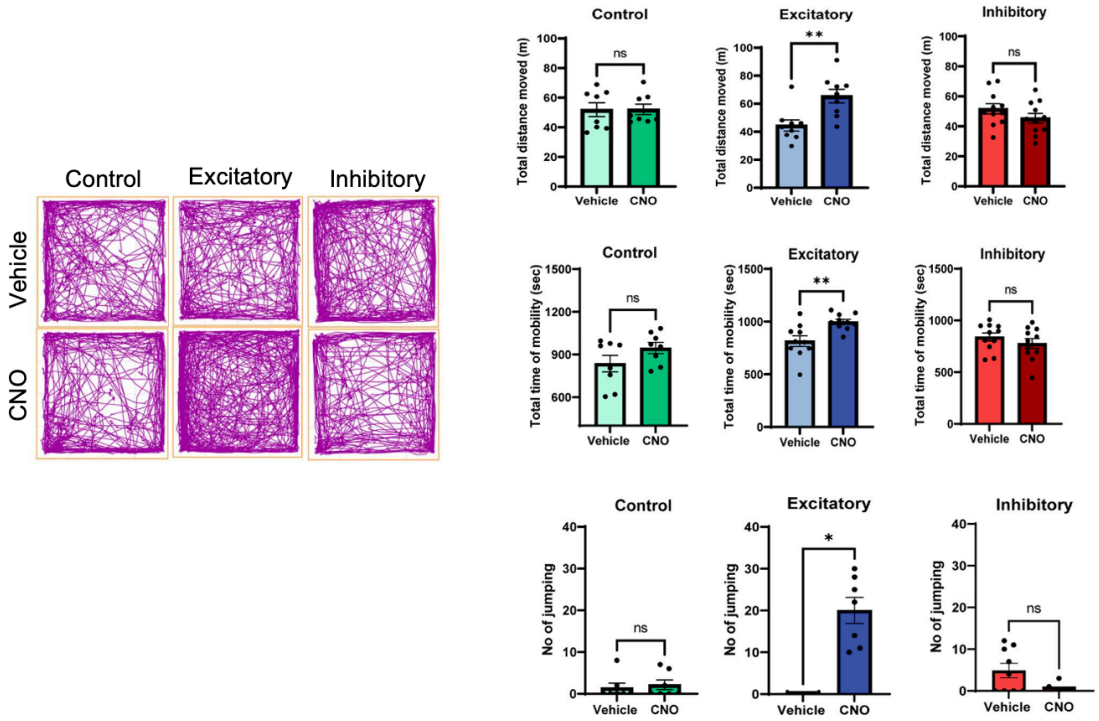

Figure 6: Chemogenetic activation of PAG^{GlyT2} neurons alters locomotion behaviour in the open field test in male mice. Plots show the position of the animals' centre point for the entire duration of the test. CNO (3 mg/kg, i.p.) administration resulted in increased total distance moved, increased mobility time, and increased jumping behaviour of GlyT2::Cre male animals injected with hM3Dq compared with vehicle (blue bars). In contrast, no significant differences were observed in locomotion behaviour when CNO (5 mg/kg, i.p.) was administered to GlyT2::Cre animals injected with hM4Di compared with vehicle (red bars). In addition, CNO (5 mg/kg, i.p.) had no effect on locomotion behaviours in GlyT2::Cre animals injected with the control mCherry vector (green bars). Individual animals are indicated on the graphs (n = 7–11) and values are presented as mean 6 SEM and significant results were determined when *p < 0.05. Figure taken from Assareh et al., 2023.

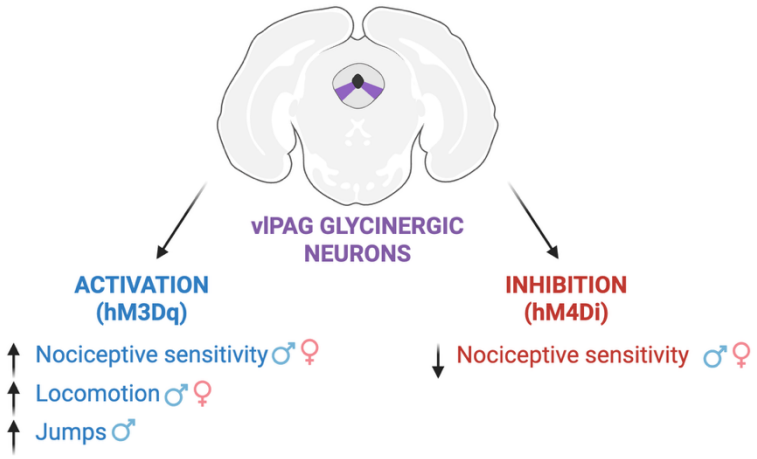

Figure 7: Summary of the role of PAG^{GlyT2} neurons. Created with BioRender.

Overall, this study found that chemogenetic modulation of PAG^{GlyT2} neurons bidirectionally modulates thermal nociception and locomotion behaviours (Figure 7). The increase in jumping responses seen in the male animals could be reflecting an active fear response (increase in mobility or jumping behaviour), suggesting that these neurons may be able to modulate anxiety-like behaviour. By demonstrating that PAG^{GlyT2} neurons can modulate nociception and locomotor behaviours, this study provided novel insights into the organisation of pain circuitry and highlights this population of neurons as a potential target for novel therapeutics.

1.4 Thesis Aims and Hypotheses

This thesis aims to address the following questions:

1. Apart from our study in the PAG, what is the current evidence supporting a role for glycine signalling in brain pain modulation?
    a. Address with a scoping review, presented in **Chapter 2**.
2. PAG^{GlyT2} neurons are uniquely restricted to the vlPAG. How does their distribution vary across the rostral-caudal axis and do these neurons co-localise with GABAergic neurons in the PAG?
    a. Address with imaging experiments, presented in **Chapter 3.**
3. Inhibitory neurons in the PAG are classically characterised as interneurons, are PAG^{GlyT2} neurons also interneurons? It has been previously demonstrated that glycinergic neurons in the PAG project to the dorsal raphe, locus coeruleus and the paraventricular nucleus of the hypothalamus, do they project to other regions outside the PAG?
    a. Address with imaging and electrophysiology experiments, presented in **Chapter 3**.
4. Chemogenetic modulation of PAG^{GlyT2} neurons bidirectionally alters nociception and locomotion responses. Their ability to modulate jumping behaviour in the open field suggests they may modulate anxiety-like behaviours and contribute to the pain experience. Can the activity of PAG^{GlyT2} neurons modulate anxiety-like behaviours?
    a. Address with chemogenetic behavioural experiments, presented in **Chapter 3.**
5. The PAG is known to modulate chronic pain states. Can chemogenetic modulation of PAG^{GlyT2} neurons in a chronic pain state alter nociception, locomotion and anxiety-like behaviours?
    a. Address with behavioural and imaging experiments, presented in **Chapter 4 and 5.**
6. There is increasing evidence that there are substantial sex differences in pain responses and pathways, including in the PAG. Are the behavioural roles of PAG^{GlyT2} neurons sex specific?
    a. Address with behavioural experiments in both males and females, presented in **Chapter 3, 4, and 5.**

It is hypothesised that glycinergic signalling in the brain can modulate pain behaviours as well as a range of other behaviours. In addition, PAG^{GlyT2} neurons are a subpopulation of GABAergic neurons in the vlPAG and contribute to other pain-related behaviours. Furthermore, it is hypothesised that the CFA model is a robust preclinical model of persistent inflammatory pain and chemogenetic inhibition of PAG^{GlyT2} neurons in a CFA state alleviates increased nociceptive sensitivity.

Overall, this project aims to increase our understanding of midbrain pain circuitry, a vital stepping stone for understanding how pain responses are organised in the mammalian brain and necessary to allow for the development of safer, more effective treatments for chronic pain (and all its complexity) in the future.

1.5 References (General Introduction)

Alikhani, V., Mohebbati, R., Hosseini, M., Khajavirad, A., & Shafei, M. N. (2021). Role of the glutamatergic system of ventrolateral periaqueductal gray (vlPAG) in the cardiovascular responses in normal and hemorrhagic conditions in rats. Iranian Journal of Basic Medical Sciences, 24(5), 586–594. https://doi.org/10.22038/ijbms.2021.53181.11978

Andermann, F., Keene, D. L., Andermann, E., & Quesney, L. F. (1980). Startle disease or hyperekplexia: Further delineation of the syndrome. Brain: A Journal of Neurology, 103(4), 985–997. https://doi.org/10.1093/brain/103.4.985

Assareh, N., Fenech, C., Power, R., Uddin, M. N., Otsu, Y., & Aubrey, K. R. (2023). Bidirectional Modulation of Nociception by GlyT2+ Neurons in the Ventrolateral Periaqueductal Gray. eNeuro, 10(6). https://doi.org/10.1523/ENEURO.0069-23.2023

Aubrey, K. R., Rossi, F. M., Ruivo, R., Alboni, S., Bellenchi, G. C., Le Goff, A., Gasnier, B., & Supplisson, S. (2007). The transporters GlyT2 and VIAAT cooperate to determine the vesicular glycinergic phenotype. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 27(23), 6273–6281. https://doi.org/10.1523/JNEUROSCI.1024-07.2007

Aubrey, K. R., & Supplisson, S. (2018). Heterogeneous Signaling at GABA and Glycine Co-releasing Terminals. Frontiers in Synaptic Neuroscience, 10. https://doi.org/10.3389/fnsyn.2018.00040

Bandler, R., & Carrive, P. (1988). Integrated defence reaction elicited by excitatory amino acid microinjection in the midbrain periaqueductal grey region of the unrestrained cat. Brain Research, 439(1), 95–106. https://doi.org/10.1016/0006-8993(88)91465-5

Bandler, R., Depaulis, A., & Vergnes, M. (1985). Indentification of midbrain neurones mediating defensive behaviour in the rat by microinjections of excitatory amino acids. Behavioural Brain Research, 15(2), 107–119. https://doi.org/10.1016/0166-4328(85)90058-0

Bao, S.-T., Rao, F., Yin, C., Niu, Y., Cao, J.-L., Xiao, C., & Zhou, C. (2025). Excitatory projections from the nucleus reuniens to the medial prefrontal cortex modulate pain and depression-like behaviors in mice. PLOS Biology, 23(5), e3003170. https://doi.org/10.1371/journal.pbio.3003170

Baptista-de-Souza, D., Tavares, L. R. R., Furuya-da-Cunha, E. M., Carneiro de Oliveira, P. E., Canto-de-Souza, L., Nunes-de-Souza, R. L., & Canto-de-Souza, A. (2020). Chronic Fluoxetine Impairs the Effects of 5-HT1A and 5-HT2C Receptors Activation in the PAG and Amygdala on Antinociception Induced by Aversive Situation in Mice. Frontiers in Pharmacology, 11. https://doi.org/10.3389/fphar.2020.00260

Behbehani, M. M. (1995). Functional characteristics of the midbrain periaqueductal gray. Progress in Neurobiology, 46(6), 575–605. https://doi.org/10.1016/0301-0082(95)00009-K

Blyth, F. M., March, L. M., Brnabic, A. J. M., Jorm, L. R., Williamson, M., & Cousins, M. J. (2001). Chronic pain in Australia: A prevalence study. PAIN, 89(2), 127. https://doi.org/10.1016/S0304-3959(00)00355-9

Bobeck, E. N., McNeal, A. L., & Morgan, M. M. (2009). Drug dependent sex-differences in periaqueducatal gray mediated antinociception in the rat. PAIN®, 147(1), 210–216. https://doi.org/10.1016/j.pain.2009.09.008

Bonica, J. J., & Hoffman, J. F. (1954). The Management of Pain with Special Emphasis on the Use of Analgesic Blocks in Diagnosis, Prognosis, and Therapy. Anesthesia & Analgesia, 34(5), 57. https://journals.lww.com/anesthesia-analgesia/citation/1954/09001/The_Management_of_Pain_with_Special_Emphasis_on.1.aspx

Cameron, A. A., Khan, I. A., Westlund, K. N., Cliffer, K. D., & Willis, W. D. (1995). The efferent projections of the periaqueductal gray in the rat: A Phaseolus vulgaris-leucoagglutinin study. I. Ascending projections. The Journal of Comparative Neurology, 351(4), 568–584. https://doi.org/10.1002/cne.903510407

Cameron, A. A., Khan, I. A., Westlund, K. N., & Willis, W. D. (1995). The efferent projections of the periaqueductal gray in the rat: A Phaseolus vulgaris-leucoagglutinin study. II. Descending projections. Journal of Comparative Neurology, 351(4), 585–601. https://doi.org/10.1002/cne.903510408

Chen, T.-W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., Schreiter, E. R., Kerr, R. A., Orger, M. B., Jayaraman, V., Looger, L. L., Svoboda, K., & Kim, D. S. (2013). Ultra-sensitive fluorescent proteins for imaging neuronal activity. Nature, 499(7458), 295–300. https://doi.org/10.1038/nature12354

Chen, Z., Lin, M.-T., Zhan, C., Zhong, N.-S., Mu, D., Lai, K.-F., & Liu, M. J. (2022). A descending pathway emanating from the periaqueductal gray mediates the development of cough-like hypersensitivity. iScience, 25(1). https://doi.org/10.1016/j.isci.2021.103641

Chéry, N., & de Koninck, Y. (1999). Junctional versus extrajunctional glycine and GABA(A) receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 19(17), 7342–7355. https://doi.org/10.1523/JNEUROSCI.19-17-07342.1999

Chronic Pain Australia. (2024). 2024 National Pain Report: The social and economic cost of chronic pain.

Cohen, H., Liu, T., Kozlovsky, N., Kaplan, Z., Zohar, J., & Mathé, A. A. (2012). The Neuropeptide Y (NPY)-ergic System is Associated with Behavioral Resilience to Stress Exposure in an Animal Model of Post-Traumatic Stress Disorder. Neuropsychopharmacology, 37(2), 350–363. https://doi.org/10.1038/npp.2011.230

De Felice, M., Sanoja, R., Wang, R., Vera-Portocarrero, L., Oyarzo, J., King, T., Ossipov, M. H., Vanderah, T. W., Lai, J., Dussor, G. O., Fields, H. L., Price, T. J., & Porreca, F. (2011). Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain. PAIN, 152(12), 2701. https://doi.org/10.1016/j.pain.2011.06.008

de Freitas, R. L., de Oliveira, R. C., de Oliveira, R., Paschoalin-Maurin, T., de Aguiar Corrêa, F. M., & Coimbra, N. C. (2014). The role of dorsomedial and ventrolateral columns of the periaqueductal gray matter and in situ 5-HT_2A and 5-HT_2C serotonergic receptors in post-ictal antinociception. Synapse (New York, N.Y.), 68(1), 16–30. https://doi.org/10.1002/syn.21697

Deisseroth, K. (2011). Optogenetics. Nature Methods, 8(1), 26–29. https://doi.org/10.1038/nmeth.f.324

Deng, H., Wu, Y., Gao, P., Kong, D., Pan, C., Xu, S., Tang, D., Jiao, Y., Wen, D., & Yu, W. (2023). Preoperative Pain Facilitates Postoperative Cognitive Dysfunction via Periaqueductal Gray Matter–Dorsal Raphe Circuit. Neuroscience, 524, 209–219. https://doi.org/10.1016/j.neuroscience.2023.03.019

Depaulis, A., Morgan, M. M., & Liebeskind, J. C. (1987). GABAergic modulation of the analgesic effects of morphine microinjected in the ventral periaqueductal gray matter of the rat. Brain Research, 436(2), 223–228. https://doi.org/10.1016/0006-8993(87)91665-9

Dominick, C. H., Blyth, F. M., & Nicholas, M. K. (2012). Unpacking the burden: Understanding the relationships between chronic pain and comorbidity in the general population. PAIN, 153(2), 293. https://doi.org/10.1016/j.pain.2011.09.018

Dufour, A., Tell, F., Kessler, J.-P., & Baude, A. (2010). Mixed GABA-glycine synapses delineate a specific topography in the nucleus tractus solitarii of adult rat. The Journal of Physiology, 588(Pt 7), 1097–1115. https://doi.org/10.1113/jphysiol.2009.184838

Dugué, G. P., Dumoulin, A., Triller, A., & Dieudonné, S. (2005). Target-dependent use of co-released inhibitory transmitters at central synapses. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 25(28), 6490–6498. https://doi.org/10.1523/JNEUROSCI.1500-05.2005

Dumoulin, A., Triller, A., & Dieudonné, S. (2001). IPSC kinetics at identified GABAergic and mixed GABAergic and glycinergic synapses onto cerebellar Golgi cells. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 21(16), 6045–6057. https://doi.org/10.1523/JNEUROSCI.21-16-06045.2001

Dworkin, R. H., O'Connor, A. B., Audette, J., Baron, R., Gourlay, G. K., Haanpää, M. L., Kent, J. L., Krane, E. J., LeBel, A. A., Levy, R. M., Mackey, S. C., Mayer, J., Miaskowski, C., Raja, S. N., Rice, A. S. C., Schmader, K. E., Stacey, B., Stanos, S., Treede, R.-D., … Wells, C. D. (2010). Recommendations for the Pharmacological Management of Neuropathic Pain: An Overview and Literature Update. Mayo Clinic Proceedings, 85(3 Suppl), S3–S14. https://doi.org/10.4065/mcp.2009.0649

Elina, K. C., Oh, B. H., Islam, J., Kim, S., & Park, Y. S. (2021). Activation of CamKIIα expressing neurons on ventrolateral periaqueductal gray improves behavioral hypersensitivity and thalamic discharge in a trigeminal neuralgia rat model. The Journal of Headache and Pain, 22(1), 47. https://doi.org/10.1186/s10194-021-01257-z

Fields, H. L., & Basbaum, A. I. (1978). Brainstem Control of Spinal Pain-Transmission Neurons. Annual Review of Physiology, 40(Volume 40, 1978), 217–248. https://doi.org/10.1146/annurev.ph.40.030178.001245

Finan, P. H., Goodin, B. R., & Smith, M. T. (2013). The Association of Sleep and Pain: An Update and a Path Forward. The Journal of Pain, 14(12), 1539–1552. https://doi.org/10.1016/j.jpain.2013.08.007

Fine, P. G. (2011). Long-Term Consequences of Chronic Pain: Mounting Evidence for Pain as a Neurological Disease and Parallels with Other Chronic Disease States. Pain Medicine, 12(7), 996–1004. https://doi.org/10.1111/j.1526-4637.2011.01187.x

Flanigan, M. E., Gianessi, C., Castle, M., Dorlean, W., Sides, T., & Kash, T. L. (2024). Bed Nucleus of the Stria Terminalis (BNST) neurons containing the serotonin 5HT2c receptor modulate operant alcohol self-administration behavior in mice. Neuropsychopharmacology, 49(4), 709–719. https://doi.org/10.1038/s41386-023-01753-7

Foster, E., Wildner, H., Tudeau, L., Haueter, S., Ralvenius, W. T., Jegen, M., Johannssen, H., Hösli, L., Haenraets, K., Ghanem, A., Conzelmann, K.-K., Bösl, M., & Zeilhofer, H. U. (2015). Targeted Ablation, Silencing, and Activation Establish Glycinergic Dorsal Horn Neurons as Key Components of a Spinal Gate for Pain and Itch. Neuron, 85(6), 1289–1304. https://doi.org/10.1016/j.neuron.2015.02.028

Frontera, J. L., Baba Aissa, H., Sala, R. W., Mailhes-Hamon, C., Georgescu, I. A., Léna, C., & Popa, D. (2020). Bidirectional control of fear memories by cerebellar neurons projecting to the ventrolateral periaqueductal grey. Nature Communications, 11(1), 5207. https://doi.org/10.1038/s41467-020-18953-0

Gao, F., Huang, J., Huang, G.-B., You, Q.-L., Yao, S., Zhao, S.-T., Liu, J., Wu, C.-H., Chen, G.-F., Liu, S.-M., Yu, Z., Zhou, Y.-L., Ning, Y.-P., Liu, S., Hu, B.-J., & Sun, X.-D. (2023). Elevated prelimbic cortex-to-basolateral amygdala circuit activity mediates comorbid anxiety-like behaviors associated with chronic pain. The Journal of Clinical Investigation, 133(9). https://doi.org/10.1172/JCI166356

Gao, Z.-R., Chen, W.-Z., Liu, M.-Z., Chen, X.-J., Wan, L., Zhang, X.-Y., Yuan, L., Lin, J.-K., Wang, M., Zhou, L., Xu, X.-H., & Sun, Y.-G. (2019). Tac1-Expressing Neurons in the Periaqueductal Gray Facilitate the Itch-Scratching Cycle via Descending Regulation. Neuron, 101(1), 45-59.e9. https://doi.org/10.1016/j.neuron.2018.11.010

Giber, K., Diana, M. A., Plattner, V., Dugué, G. P., Bokor, H., Rousseau, C. V., Maglóczky, Z., Havas, L., Hangya, B., Wildner, H., Zeilhofer, H. U., Dieudonné, S., & Acsády, L. (2015). A subcortical inhibitory signal for behavioral arrest in the thalamus. Nature Neuroscience, 18(4), 562–568. https://doi.org/10.1038/nn.3951

Gomeza, J., Ohno, K., Hülsmann, S., Armsen, W., Eulenburg, V., Richter, D. W., Laube, B., & Betz, H. (2003). Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. Neuron, 40(4), 797–806. https://doi.org/10.1016/s0896-6273(03)00673-1

Guo, Y., Song, Y., Cao, F., Li, A., Hao, X., Shi, W., Zhou, Z., Cao, J., Liu, Y., Mi, W., & Tong, L. (2023). Ventrolateral periaqueductal gray GABAergic neurons promote arousal of sevoflurane anesthesia through cortico-midbrain circuit. iScience, 26(9), 107486. https://doi.org/10.1016/j.isci.2023.107486

Hao, S., Yang, H., Wang, X., He, Y., Xu, H., Wu, X., Pan, L., Liu, Y., Lou, H., Xu, H., Ma, H., Xi, W., Zhou, Y., Duan, S., & Wang, H. (2019). The Lateral Hypothalamic and BNST GABAergic Projections to the Anterior Ventrolateral Periaqueductal Gray Regulate Feeding. Cell Reports, 28(3), 616-624.e5. https://doi.org/10.1016/j.celrep.2019.06.051

Harvey, R. J., Depner, U. B., Wässle, H., Ahmadi, S., Heindl, C., Reinold, H., Smart, T. G., Harvey, K., Schütz, B., Abo-Salem, O. M., Zimmer, A., Poisbeau, P., Welzl, H., Wolfer, D. P., Betz, H., Zeilhofer, H. U., & Müller, U. (2004). GlyR alpha3: An essential target for spinal PGE2-mediated inflammatory pain sensitization. Science (New York, N.Y.), 304(5672), 884–887. https://doi.org/10.1126/science.1094925

Honshuku, Y., Yamaguchi, Y., Kawazoe, K., Nishida, Y., Kurauchi, Y., Minami, M., Katsuki, H., & Hitora-Imamura, N. (2025). Functional analysis of periaqueductal gray neurons projecting to the medulla in active and passive defensive behaviors. Neuroscience Research, 218, 104939. https://doi.org/10.1016/j.neures.2025.104939

Hu, Z., Mu, Y., Huang, L., Hu, Y., Chen, Z., Yang, Y., Huang, X., Fu, Y., Xi, Y., Lin, S., Tao, Q., Xu, F., So, K.-F., & Ren, C. (2022). A visual circuit related to the periaqueductal gray area for the antinociceptive effects of bright light treatment. Neuron, 110(10), 1712-1727.e7. https://doi.org/10.1016/j.neuron.2022.02.009

Huang, J., Gadotti, V. M., Chen, L., Souza, I. A., Huang, S., Wang, D., Ramakrishnan, C., Deisseroth, K., Zhang, Z., & Zamponi, G. W. (2019). A neuronal circuit for activating descending modulation of neuropathic pain. Nature Neuroscience, 22(10), 1659–1668. https://doi.org/10.1038/s41593-019-0481-5

Huang, J., Zhang, Z., Gambeta, E., Chen, L., & Zamponi, G. W. (2021). An orbitofrontal cortex to midbrain projection modulates hypersensitivity after peripheral nerve injury. Cell Reports, 35(4), 109033. https://doi.org/10.1016/j.celrep.2021.109033

Huang, S., Zhang, Z., Gambeta, E., Xu, S. C., Thomas, C., Godfrey, N., Chen, L., M'Dahoma, S., Borgland, S. L., & Zamponi, G. W. (2020). Dopamine Inputs from the Ventral Tegmental Area into the Medial Prefrontal Cortex Modulate Neuropathic Pain-Associated Behaviors in Mice. Cell Reports, 31(12), 107812. https://doi.org/10.1016/j.celrep.2020.107812

Islam, J., Rahman, M. T., Ali, M., Kim, H. K., KC, E., & Park, Y. S. (2025). Optogenetic inhibition of ventrolateral orbitofrontal cortex astrocytes facilitates ventrolateral periaqueductal gray glutamatergic activity to reduce hypersensitivity in infraorbital nerve injury rat model. The Journal of Headache and Pain, 26(1), 41. https://doi.org/10.1186/s10194-025-01977-6

Iwasaki, M., Lefevre, A., Althammer, F., Clauss Creusot, E., Łapieś, O., Petitjean, H., Hilfiger, L., Kerspern, D., Melchior, M., Küppers, S., Krabichler, Q., Patwell, R., Kania, A., Gruber, T., Kirchner, M. K., Wimmer, M., Fröhlich, H., Dötsch, L., Schimmer, J., … Charlet, A. (2023). An analgesic pathway from parvocellular oxytocin neurons to the periaqueductal gray in rats. Nature Communications, 14, 1066. https://doi.org/10.1038/s41467-023-36641-7

Jiang, Z., Wang, Q., Zhao, J., Wang, J., Li, Y., Dai, W., Zhang, X., Fang, Z., Hou, W., & Xiong, L. (2022). Sex-specific cannabinoid 1 receptors on GABAergic neurons in the ventrolateral periaqueductal gray mediate analgesia in mice. Journal of Comparative Neurology, 530(13), 2315–2334. https://doi.org/10.1002/cne.25334

Jonas, P., Bischofberger, J., & Sandkühler, J. (1998). Corelease of Two Fast Neurotransmitters at a Central Synapse. Science, 281(5375), 419–424. https://doi.org/10.1126/science.281.5375.419

Keay, K. A., & Bandler, R. (2015). Chapter 10—Periaqueductal Gray. In G. Paxinos (Ed.), The Rat Nervous System (Fourth Edition) (pp. 207–221). Academic Press. https://doi.org/10.1016/B978-0-12-374245-2.00010-3

Keay, K., & Bandler, R. (2008). 5.42—Emotional and Behavioral Significance of the Pain Signal and the Role of the Midbrain Periaqueductal Gray (PAG). In R. H. Masland, T. D. Albright, T. D. Albright, R. H. Masland, P. Dallos, D. Oertel, S. Firestein, G. K. Beauchamp, M. Catherine Bushnell, A. I. Basbaum, J. H. Kaas, & E. P. Gardner (Eds.), The

Senses: A Comprehensive Reference (pp. 627–634). Academic Press. https://doi.org/10.1016/B978-012370880-9.00184-5

Keller, A. F., Coull, J. A., Chery, N., Poisbeau, P., & De Koninck, Y. (2001). Region-specific developmental specialization of GABA-glycine cosynapses in laminas I-II of the rat spinal dorsal horn. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 21(20), 7871–7880. https://doi.org/10.1523/JNEUROSCI.21-20-07871.2001

Kimmey, B. A., Ejoh, L., Shangloo, L., Wojick, J. A., Chehimi, S. N., McCall, N. M., Oswell, C. S., Mahmood, M., Yang, L., Samineni, V. K., Ramakrishnan, C., Deisseroth, K., Crist, R. C., Reiner, B. C., Tian, L., & Corder, G. (2025). Convergent state-control of endogenous opioid analgesia. bioRxiv, 2025.01.03.631111. https://doi.org/10.1101/2025.01.03.631111

Kosten, T. R., & George, T. P. (2002). The Neurobiology of Opioid Dependence: Implications for Treatment. Science & Practice Perspectives, 1(1), 13–20. https://doi.org/10.1151/spp021113

Kroeger, D., Bandaru, S. S., Madara, J. C., & Vetrivelan, R. (2019). Ventrolateral periaqueductal gray mediates rapid eye movement sleep regulation by melanin-concentrating hormone neurons. Neuroscience, 406, 314–324. https://doi.org/10.1016/j.neuroscience.2019.03.020

Lau, B. K., & Vaughan, C. W. (2014). Descending modulation of pain: The GABA disinhibition hypothesis of analgesia. Current Opinion in Neurobiology, 29, 159–164. https://doi.org/10.1016/j.conb.2014.07.010

Laurent, R. S., Damonte, V. M., Tsuda, A. C., & Kauer, J. A. (2020). Periaqueductal Gray and Rostromedial Tegmental Inhibitory Afferents to VTA Have Distinct Synaptic Plasticity and Opiate Sensitivity. Neuron, 106(4), 624-636.e4. https://doi.org/10.1016/j.neuron.2020.02.029

La-Vu, M. Q., Sethi, E., Maesta-Pereira, S., Schuette, P. J., Tobias, B. C., Reis, F. M., Wang, W., Torossian, A., Bishop, A., Leonard, S. J., Lin, L., Cahill, C. M., & Adhikari, A. (2022). Sparse genetically defined neurons refine the canonical role of periaqueductal gray columnar organization. eLife, 11, e77115. https://doi.org/10.7554/eLife.77115

Lawrenson, C., Paci, E., Pickford, J., Drake, R. A., Lumb, B. M., & Apps, R. (2021). Cerebellar modulation of memory encoding in the periaqueductal grey and fear behaviour. eLife, 11, e76278. https://doi.org/10.7554/eLife.76278

Lee, M. T., Peng, W.-H., Wu, C.-C., Kan, H.-W., Wang, D.-W., Teng, Y.-N., & Ho, Y.-C. (2023). Impaired Ventrolateral Periaqueductal Gray-Ventral Tegmental area Pathway Contributes to Chronic Pain-Induced Depression-Like Behavior in Mice. Molecular Neurobiology, 60(10), 5708–5724. https://doi.org/10.1007/s12035-023-03439-z

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, 41(8), 2122–2132. https://doi.org/10.1038/npp.2016.12

Li, D., Mai, J.-W., Deng, J., Chen, L., Fan, H.-T., Zhang, W.-L., Xin, W.-J., Feng, X., Xu, T., & Luo, D.-X. (2024). A neural circuit from thalamic paraventricular nucleus via zona incerta to periaqueductal gray for the facilitation of neuropathic pain. Neurobiology of Disease, 202, 106699. https://doi.org/10.1016/j.nbd.2024.106699

Li, J.-N., Ren, J.-H., He, C.-B., Zhao, W.-J., Li, H., Dong, Y.-L., & Li, Y.-Q. (2021). Projections from the lateral parabrachial nucleus to the lateral and ventral lateral periaqueductal gray subregions mediate the itching sensation. Pain, 162(6), 1848–1863. https://doi.org/10.1097/j.pain.0000000000002193

Li, Y., Zeng, J., Zhang, J., Yue, C., Zhong, W., Liu, Z., Feng, Q., & Luo, M. (2018). Hypothalamic Circuits for Predation and Evasion. Neuron, 97(4), 911-924.e5. https://doi.org/10.1016/j.neuron.2018.01.005

Liang, S.-H., Zhao, W.-J., Yin, J.-B., Chen, Y.-B., Li, J.-N., Feng, B., Lu, Y.-C., Wang, J., Dong, Y.-L., & Li, Y.-Q. (2020). A Neural Circuit from Thalamic Paraventricular Nucleus to Central Amygdala for the Facilitation of Neuropathic Pain. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 40(41), 7837–7854. https://doi.org/10.1523/JNEUROSCI.2487-19.2020

Libenson, M. H., & Yang, J. M. (2001). Case 12-2001—A 16-Year-Old Boy with an Altered Mental Status and Muscle Rigidity. New England Journal of Medicine, 344(16), 1232–1239. https://doi.org/10.1056/NEJM200104193441608

Linnman, C., Beucke, J.-C., Jensen, K. B., Gollub, R. L., & Kong, J. (2012). Sex similarities and differences in pain-related periaqueductal gray connectivity. Pain, 153(2), 444–454. https://doi.org/10.1016/j.pain.2011.11.006

Liu, X., He, J., Jiang, W., Wen, S., & Xiao, Z. (2023). The Roles of Periaqueductal Gray and Dorsal Raphe Nucleus Dopaminergic Systems in the Mechanisms of Thermal Hypersensitivity and Depression in Mice. The Journal of Pain, 24(7), 1213–1228. https://doi.org/10.1016/j.jpain.2023.02.004

Lowery-Gionta, E. G., DiBerto, J., Mazzone, C. M., & Kash, T. L. (2018). GABA neurons of the ventral periaqueductal gray area modulate behaviors associated with anxiety and conditioned fear. Brain Structure and Function, 223(8), 3787–3799. https://doi.org/10.1007/s00429-018-1724-z

Loyd, D. R., Morgan, M. M., & Murphy, A. Z. (2007). Morphine preferentially activates the periaqueductal gray–rostral ventromedial medullary pathway in the male rat: A potential mechanism for sex differences in antinociception. Neuroscience, 147(2), 456–468. https://doi.org/10.1016/j.neuroscience.2007.03.053

Lu, Y., Dong, H., Gao, Y., Gong, Y., Ren, Y., Gu, N., Zhou, S., Xia, N., Sun, Y.-Y., Ji, R.-R., & Xiong, L. (2013). A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. The Journal of Clinical Investigation, 123(9), 4050–4062. https://doi.org/10.1172/JCI70026

Millan, M. J. (2002). Descending control of pain. Progress in Neurobiology, 66(6), 355–474. https://doi.org/10.1016/s0301-0082(02)00009-6

Millington, W. R., Yilmaz, M. S., & Feleder, C. (2016). The initial fall in arterial pressure evoked by endotoxin is mediated by the ventrolateral periaqueductal gray. Clinical and Experimental Pharmacology and Physiology, 43(6), 612–615. https://doi.org/10.1111/1440-1681.12573

Mogil, J. S. (2012). Sex differences in pain and pain inhibition: Multiple explanations of a controversial phenomenon. Nature Reviews Neuroscience, 13(12), 859–866. https://doi.org/10.1038/nrn3360

Moreau, J. L., & Fields, H. L. (1986). Evidence for GABA involvement in midbrain control of medullary neurons that modulate nociceptive transmission. Brain Research, 397(1), 37–46. https://doi.org/10.1016/0006-8993(86)91367-3

Nabekura, J., Katsurabayashi, S., Kakazu, Y., Shibata, S., Matsubara, A., Jinno, S., Mizoguchi, Y., Sasaki, A., & Ishibashi, H. (2004). Developmental switch from GABA to glycine release in single central synaptic terminals. Nature Neuroscience, 7(1), 17–23. https://doi.org/10.1038/nn1170

Neyama, H., Wu, Y., Nakaya, Y., Kato, S., Shimizu, T., Tahara, T., Shigeta, M., Inoue, M., Miyamichi, K., Matsushita, N., Mashimo, T., Miyasaka, Y., Dai, Y., Noguchi, K., Watanabe, Y., Kobayashi, M., Kobayashi, K., & Cui, Y. (2025). Opioidergic activation of the descending pain inhibitory system underlies placebo analgesia. Science Advances, 11(3), eadp8494. https://doi.org/10.1126/sciadv.adp8494

Obray, J. D., Wilkes, E. T., Scofield, M., & Chandler, L. J. (2025). Adolescent alcohol exposure promotes mechanical allodynia and alters synaptic function at inputs from the basolateral amygdala to the prelimbic cortex. eLife, 13, RP101667. https://doi.org/10.7554/eLife.101667

Ou, C., Zhang, K., Mu, Y., Huang, Z., Li, X., Huang, W., Wang, Y., Zeng, W., & Ouyang, H. (2024). YTHDF1 in periaqueductal gray inhibitory neurons contributes to morphine withdrawal responses in mice. BMC Medicine, 22(1), 406. https://doi.org/10.1186/s12916-024-03634-2

Pati, D., Downs, A. M., McElligott, Z. A., & Kash, T. L. (2023). Chronic Ethanol Exposure Modulates Periaqueductal Gray to Extended Amygdala Dopamine Circuit. Journal of Neuroscience, 43(5), 709–721. https://doi.org/10.1523/JNEUROSCI.1219-22.2022

Peiser-Oliver, J. M., Evans, S., Adams, D. J., Christie, M. J., Vandenberg, R. J., & Mohammadi, S. A. (2022). Glycinergic Modulation of Pain in Behavioral Animal Models. Frontiers in Pharmacology, 13, 860903. https://doi.org/10.3389/fphar.2022.860903

Porter-Stransky, K. A., Centanni, S. W., Karne, S. L., Odil, L. M., Fekir, S., Wong, J. C., Jerome, C., Mitchell, H. A., Escayg, A., Pedersen, N. P., Winder, D. G., Mitrano, D. A., & Weinshenker, D. (2019). Noradrenergic Transmission at Alpha1-Adrenergic Receptors in the Ventral Periaqueductal Gray Modulates Arousal. Biological Psychiatry, 85(3), 237–247. https://doi.org/10.1016/j.biopsych.2018.07.027

Price, D. D. (2000). Psychological and Neural Mechanisms of the Affective Dimension of Pain. Science, 288(5472), 1769–1772. https://doi.org/10.1126/science.288.5472.1769

Rácz, I., Schütz, B., Abo-Salem, O. M., & Zimmer, A. (2005). Visceral, inflammatory and neuropathic pain in glycine receptor alpha 3-deficient mice. Neuroreport, 16(18), 2025–2028. https://doi.org/10.1097/00001756-200512190-00011

Raja, S. N., Carr, D. B., Cohen, M., Finnerup, N. B., Flor, H., Gibson, S., Keefe, F. J., Mogil, J. S., Ringkamp, M., Sluka, K. A., Song, X.-J., Stevens, B., Sullivan, M. D., Tutelman, P. R., Ushida, T., & Vader, K. (2020). The revised

International Association for the Study of Pain definition of pain: Concepts, challenges, and compromises. PAIN, 161(9), 1976. https://doi.org/10.1097/j.pain.0000000000001939

Rampon, C., Luppi, P. H., Fort, P., Peyron, C., & Jouvet, M. (1996). Distribution of glycine-immunoreactive cell bodies and fibers in the rat brain. Neuroscience, 75(3), 737–755. https://doi.org/10.1016/0306-4522(96)00278-3

Rampon, C., Peyron, C., Gervasoni, D., Pow, D. V., Luppi, P.-H., & Fort, P. (1999). Origins of the glycinergic inputs to the rat locus coeruleus and dorsal raphe nuclei: A study combining retrograde tracing with glycine immunohistochemistry. European Journal of Neuroscience, 11(3), 1058–1066. https://doi.org/10.1046/j.1460-9568.1999.00511.x

Rao, Y., Gao, Z., Li, X., Li, X., Li, J., Liang, S., Li, D., Zhai, J., Yan, J., Yao, J., & Chen, X. (2022). Ventrolateral Periaqueductal Gray Neurons Are Active During Urination. Frontiers in Cellular Neuroscience, 16. https://doi.org/10.3389/fncel.2022.865186

Reis, F. M. C. V., Maesta-Pereira, S., Ollivier, M., Schuette, P. J., Sethi, E., Miranda, B. A., Iniguez, E., Chakerian, M., Vaughn, E., Sehgal, M., Nguyen, D. C. T., Yuan, F. T. H., Torossian, A., Ikebara, J. M., Kihara, A. H., Silva, A. J., Kao, J. C., Khakh, B. S., & Adhikari, A. (2024). Control of feeding by a bottom-up midbrain-subthalamic pathway. Nature Communications, 15(1), 2111. https://doi.org/10.1038/s41467-024-46430-5

Roman-Ortiz, C., Guevara, J. A., & Clem, R. L. (2021). GABAergic basal forebrain projections to the periaqueductal gray promote food consumption, reward and predation. Scientific Reports, 11(1), 22638. https://doi.org/10.1038/s41598-021-02157-7

Rossier, D., La Franca, V., Salemi, T., Natale, S., & Gross, C. T. (2021). A neural circuit for competing approach and defense underlying prey capture. Proceedings of the National Academy of Sciences, 118(15), e2013411118. https://doi.org/10.1073/pnas.2013411118

Roth, B. L. (2016). DREADDs for Neuroscientists. Neuron, 89(4), 683–694. https://doi.org/10.1016/j.neuron.2016.01.040

Rousseau, F., Aubrey, K. R., & Supplisson, S. (2008). The glycine transporter GlyT2 controls the dynamics of synaptic vesicle refilling in inhibitory spinal cord neurons. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 28(39), 9755–9768. https://doi.org/10.1523/JNEUROSCI.0509-08.2008

Rozeske, R. R., Jercog, D., Karalis, N., Chaudun, F., Khoder, S., Girard, D., Winke, N., & Herry, C. (2018). Prefrontal-Periaqueductal Gray-Projecting Neurons Mediate Context Fear Discrimination. Neuron, 97(4), 898-910.e6. https://doi.org/10.1016/j.neuron.2017.12.044

Samineni, V. K., Grajales-Reyes, J. G., Copits, B. A., O'Brien, D. E., Trigg, S. L., Gomez, A. M., Bruchas, M. R., & Gereau, R. W. (2017). Divergent Modulation of Nociception by Glutamatergic and GABAergic Neuronal Subpopulations in the Periaqueductal Gray. eNeuro, 4(2). https://doi.org/10.1523/ENEURO.0129-16.2017

Samineni, V. K., Grajales-Reyes, J. G., Sundaram, S. S., Yoo, J. J., & Gereau, R. W. (2019). Cell type-specific modulation of sensory and affective components of itch in the periaqueductal gray. Nature Communications, 10(1), 4356. https://doi.org/10.1038/s41467-019-12316-0

Shah, D. P., Chaudhury, Y., & Barik, A. (2025). Acute restraint stress and pain modulation depend on the interaction between the periaqueductal gray and the lateral septum. Journal of Neuroscience. https://doi.org/10.1523/JNEUROSCI.2257-24.2025

Siemian, J. N., Arenivar, M. A., Sarsfield, S., Borja, C. B., Erbaugh, L. J., Eagle, A. L., Robison, A. J., Leinninger, G., & Aponte, Y. (2021). An excitatory lateral hypothalamic circuit orchestrating pain behaviors in mice. eLife, 10, e66446. https://doi.org/10.7554/eLife.66446

Skog, T. D., Johnson, S. B., Hinz, D. C., Lingg, R. T., Schulz, E. N., Luna, J. T., Beltz, T. G., Romig-Martin, S. A., Gantz, S. C., Xue, B., Johnson, A. K., & Radley, J. J. (2024). A Prefrontal→Periaqueductal Gray Pathway Differentially Engages Autonomic, Hormonal, and Behavioral Features of the Stress-Coping Response. The Journal of Neuroscience, 44(46), e0844242024. https://doi.org/10.1523/JNEUROSCI.0844-24.2024

Sun, F., Zhou, J., Dai, B., Qian, T., Zeng, J., Li, X., Zhuo, Y., Zhang, Y., Wang, Y., Qian, C., Tan, K., Feng, J., Dong, H., Lin, D., Cui, G., & Li, Y. (2020). Next-generation GRAB sensors for monitoring dopaminergic activity in vivo. Nature Methods, 17(11), 1156–1166. https://doi.org/10.1038/s41592-020-00981-9

Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J.-I., Obata, K., & Kaneko, T. (2003). Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. The Journal of Comparative Neurology, 467(1), 60–79. https://doi.org/10.1002/cne.10905

Tan, N., Shi, J., Xu, L., Zheng, Y., Wang, X., Lai, N., Fang, Z., Chen, J., Wang, Y., & Chen, Z. (2022). Lateral Hypothalamus Calcium/Calmodulin-Dependent Protein Kinase II α Neurons Encode Novelty-Seeking Signals to Promote Predatory Eating. Research, 2022. https://doi.org/10.34133/2022/9802382

Tanaka, I., & Ezure, K. (2004). Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. Neuroscience Research, 49(2), 165–178. https://doi.org/10.1016/j.neures.2004.02.007

Taylor, N. E., Pei, J., Zhang, J., Vlasov, K. Y., Davis, T., Taylor, E., Weng, F.-J., Dort, C. J. V., 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). https://doi.org/10.1523/ENEURO.0018-18.2019

Tonsfeldt, K. J., Suchland, K. L., Beeson, K. A., Lowe, J. D., Li, M., & Ingram, S. L. (2016). Sex Differences in GABAA Signaling in the Periaqueductal Gray Induced by Persistent Inflammation. Journal of Neuroscience, 36(5), 1669–1681. https://doi.org/10.1523/JNEUROSCI.1928-15.2016

Tovote, P., Esposito, M. S., Botta, P., Chaudun, F., Fadok, J. P., Markovic, M., Wolff, S. B. E., Ramakrishnan, C., Fenno, L., Deisseroth, K., Herry, C., Arber, S., & Lüthi, A. (2016). Midbrain circuits for defensive behaviour. Nature, 534(7606), 206–212. https://doi.org/10.1038/nature17996

Treede, R.-D., Rief, W., Barke, A., Aziz, Q., Bennett, M. I., Benoliel, R., Cohen, M., Evers, S., Finnerup, N. B., First, M. B., Giamberardino, M. A., Kaasa, S., Korwisi, B., Kosek, E., Lavand'homme, P., Nicholas, M., Perrot, S., Scholz, J., Schug, S., … Wang, S.-J. (2019). Chronic pain as a symptom or a disease: The IASP Classification of Chronic Pain

for the International Classification of Diseases (ICD-11). PAIN, 160(1), 19. https://doi.org/10.1097/j.pain.0000000000001384

Treede, R.-D., Rief, W., Barke, A., Aziz, Q., Bennett, M. I., Benoliel, R., Cohen, M., Evers, S., Finnerup, N. B., First, M. B., Giamberardino, M. A., Kaasa, S., Kosek, E., Lavand'homme, P., Nicholas, M., Perrot, S., Scholz, J., Schug, S., Smith, B. H., … Wang, S.-J. (2015). A classification of chronic pain for ICD-11. PAIN, 156(6), 1003. https://doi.org/10.1097/j.pain.0000000000000160

Trevizan-Baú, P., Furuya, W. I., Mazzone, S. B., Stanić, D., Dhingra, R. R., & Dutschmann, M. (2021). Reciprocal connectivity of the periaqueductal gray with the ponto-medullary respiratory network in rat. Brain Research, 1757, 147255. https://doi.org/10.1016/j.brainres.2020.147255

Ullah, F., dos Anjos-Garcia, T., Mendes-Gomes, J., Elias-Filho, D. H., Falconi-Sobrinho, L. L., Freitas, R. L. de, Khan, A. U., Oliveira, R. de, & Coimbra, N. C. (2017). Connexions between the dorsomedial division of the ventromedial hypothalamus and the dorsal periaqueductal grey matter are critical in the elaboration of hypothalamically mediated panic-like behaviour. Behavioural Brain Research, 319, 135–147. https://doi.org/10.1016/j.bbr.2016.11.026

Vaaga, C. E., Brown, S. T., & Raman, I. M. (2020). Cerebellar modulation of synaptic input to freezing-related neurons in the periaqueductal gray. eLife, 9, e54302. https://doi.org/10.7554/eLife.54302

van den Pol, A. N., & Gorcs, T. (1988). Glycine and glycine receptor immunoreactivity in brain and spinal cord. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 8(2), 472–492. https://doi.org/10.1523/JNEUROSCI.08-02-00472.1988

Varga, E., Farkas, E., Zséli, G., Kádár, A., Venczel, A., Kővári, D., Németh, D., Máté, Z., Erdélyi, F., Horváth, A., Szenci, O., Watanabe, M., Lechan, R. M., Gereben, B., & Fekete, C. (2019). Thyrotropin-Releasing-Hormone-Synthesizing Neurons of the Hypothalamic Paraventricular Nucleus Are Inhibited by Glycinergic Inputs. Thyroid: Official Journal of the American Thyroid Association, 29(12), 1858–1868. https://doi.org/10.1089/thy.2019.0357

Vaughan, C. W., Ingram, S. L., Connor, M. A., & Christie, M. J. (1997). How opioids inhibit GABA-mediated neurotransmission. Nature, 390(6660), 611–614. https://doi.org/10.1038/37610

Vaughn, E., Eichhorn, S., Jung, W., Zhuang, X., & Dulac, C. (2022). Three-dimensional Interrogation of Cell Types and Instinctive Behavior in the Periaqueductal Gray (p. 2022.06.27.497769). bioRxiv. https://doi.org/10.1101/2022.06.27.497769

Vuilleumier, P. H., Fritsche, R., Schliessbach, J., Schmitt, B., Arendt-Nielsen, L., Zeilhofer, H. U., & Curatolo, M. (2018). Mutations affecting glycinergic neurotransmission in hyperekplexia increase pain sensitivity. Brain: A Journal of Neurology, 141(1), 63–71. https://doi.org/10.1093/brain/awx289

Wang, S., Yang, Y., Hao, S., Sun, Y., & Wang, H. (2025). Glutamatergic Periaqueductal Gray Projections to the Locus Coeruleus Orchestrate Adaptive Arousal States in Threatening Contexts. Neuroscience Bulletin. https://doi.org/10.1007/s12264-025-01491-4

Wang, X., Wu, X., Wu, H., Xiao, H., Hao, S., Wang, B., Li, C., Bleymehl, K., Kauschke, S. G., Mack, V., Ferger, B., Klein, H., Zheng, R., Duan, S., & Wang, H. (2023). Neural adaption in midbrain GABAergic cells contributes to high-fat diet–induced obesity. Science Advances, 9(44), eadh2884. https://doi.org/10.1126/sciadv.adh2884

Waung, M. W., Margolis, E. B., Charbit, A. R., & Fields, H. L. (2019). A Midbrain Circuit that Mediates Headache Aversiveness in Rats. Cell Reports, 28(11), 2739-2747.e4. https://doi.org/10.1016/j.celrep.2019.08.009

Weber, F., Chung, S., Beier, K. T., Xu, M., Luo, L., & Dan, Y. (2015). Control of REM sleep by ventral medulla GABAergic neurons. Nature, 526(7573), 435–438. https://doi.org/10.1038/nature14979

Weber, F., Hoang Do, J. P., Chung, S., Beier, K. T., Bikov, M., Saffari Doost, M., & Dan, Y. (2018). Regulation of REM and Non-REM Sleep by Periaqueductal GABAergic Neurons. Nature Communications, 9(1), 354. https://doi.org/10.1038/s41467-017-02765-w

Winke, N., Aby, F., Jercog, D., Bienvenu, T., Riffault, C., Bouali-Benazzouz, R., Viellard, J., Girard, D., Grivet, Z., Landry, M., Castell, L., Valjent, E., Valerio, S., Fossat, P., & Herry, C. (2025). Midbrain somatostatin-expressing cells control pain-suppression during defensive states. eLife, 14. https://doi.org/10.7554/eLife.106826.1

Winters, B. L., Lau, B. K., & Vaughan, C. W. (2022). Cannabinoids and Opioids Differentially Target Extrinsic and Intrinsic GABAergic Inputs onto the Periaqueductal Grey Descending Pathway. Journal of Neuroscience, 42(41), 7744–7756. https://doi.org/10.1523/JNEUROSCI.0997-22.2022

Wojcik, S. M., Katsurabayashi, S., Guillemin, I., Friauf, E., Rosenmund, C., Brose, N., & Rhee, J.-S. (2006). A shared vesicular carrier allows synaptic corelease of GABA and glycine. Neuron, 50(4), 575–587. https://doi.org/10.1016/j.neuron.2006.04.016

Xie, L., Wu, H., Chen, Q., Xu, F., Li, H., Xu, Q., Jiao, C., Sun, L., Ullah, R., & Chen, X. (2023). Divergent modulation of pain and anxiety by GABAergic neurons in the ventrolateral periaqueductal gray and dorsal raphe. Neuropsychopharmacology, 48(10), 1509–1519. https://doi.org/10.1038/s41386-022-01520-0

Xie, Z., Gu, H., Huang, M., Cheng, X., Shang, C., Tao, T., Li, D., Xie, Y., Zhao, J., Lu, W., Zhang, Z., Zhan, C., Tang, Z., Zhang, F., & Cao, P. (2022). Mechanically evoked defensive attack is controlled by GABAergic neurons in the anterior hypothalamic nucleus. Nature Neuroscience, 25(1), 72–85. https://doi.org/10.1038/s41593-021-00985-4

Yaksh, T. L. (1989). Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: Effects of modulatory receptor systems and excitatory amino acid antagonists. Pain, 37(1), 111–123. https://doi.org/10.1016/0304-3959(89)90160-7

Yamarick, W., Walson, P., & DiTraglia, J. (1992). Strychnine poisoning in an adolescent. Journal of Toxicology. Clinical Toxicology, 30(1), 141–148. https://doi.org/10.3109/15563659208994455

Yan, C., & Liu, Z. (2024). The role of periaqueductal gray astrocytes in anxiety-like behavior induced by acute stress. Biochemical and Biophysical Research Communications, 720, 150073. https://doi.org/10.1016/j.bbrc.2024.150073

Yan, J., Gao, Z., Li, X., Li, J., Yuan, C., Liang, S., Li, J., Deng, G., Yin, L., Pang, S., Shao, W., Xia, X., Zhu, H., & Yao, J. (2025). SST neurons in the periaqueductal gray regulate urination and bladder function. Communications Biology, 8(1), 639. https://doi.org/10.1038/s42003-025-08069-w

Yang, L., Lu, J., Guo, J., Chen, J., Xiong, F., Wang, X., Chen, L., & Yu, C. (2022). Ventrolateral Periaqueductal Gray Astrocytes Regulate Nociceptive Sensation and Emotional Motivation in Diabetic Neuropathic Pain. Journal of Neuroscience, 42(43), 8184–8199. https://doi.org/10.1523/JNEUROSCI.0920-22.2022

Yin, J.-B., Liang, S.-H., Li, F., Zhao, W.-J., Bai, Y., Sun, Y., Wu, Z.-Y., Ding, T., Sun, Y., Liu, H.-X., Lu, Y.-C., Zhang, T., Huang, J., Chen, T., Li, H., Chen, Z.-F., Cao, J., Ren, R., Peng, Y.-N., … Li, Y.-Q. (2020). dmPFC-vlPAG projection neurons contribute to pain threshold maintenance and antianxiety behaviors. The Journal of Clinical Investigation, 130(12), 6555–6570. https://doi.org/10.1172/JCI127607

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

Zafra, F., Ibáñez, I., & Giménez, C. (2016). Glycinergic transmission: Glycine transporter GlyT2 in neuronal pathologies. Neuronal Signaling, 1(1), NS20160009. https://doi.org/10.1042/NS20160009

Zeilhofer, H. U. (2005). The glycinergic control of spinal pain processing. Cellular and Molecular Life Sciences: CMLS, 62(18), 2027–2035. https://doi.org/10.1007/s00018-005-5107-2

Zeilhofer, H. U., Studler, B., Arabadzisz, D., Schweizer, C., Ahmadi, S., Layh, B., Bösl, M. R., & Fritschy, J.-M. (2005). Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. The Journal of Comparative Neurology, 482(2), 123–141. https://doi.org/10.1002/cne.20349

Zhang, G.-W., Shen, L., Tao, C., Jung, A.-H., Peng, B., Li, Z., Zhang, L. I., & Tao, H. W. (2021). Medial preoptic area antagonistically mediates stress-induced anxiety and parental behavior. Nature Neuroscience, 24(4), 516–528. https://doi.org/10.1038/s41593-020-00784-3

Zhang, H., Zhu, Z., Ma, W.-X., Kong, L.-X., Yuan, P.-C., Bu, L.-F., Han, J., Huang, Z.-L., & Wang, Y.-Q. (2024). The contribution of periaqueductal gray in the regulation of physiological and pathological behaviors. Frontiers in Neuroscience, 18, 1380171. https://doi.org/10.3389/fnins.2024.1380171

Zhang, J., Wang, L., Yang, Y., Wang, S., Huang, C., Yang, L., Li, B., Wang, L., Wang, H., & Hao, S. (2023). Dissection of the bed nucleus of the stria terminalis neuronal subtypes in feeding regulation. Physiology & Behavior, 271, 114333. https://doi.org/10.1016/j.physbeh.2023.114333

Zhang, Y., Huang, X., Xin, W.-J., He, S., Deng, J., & Ruan, X. (2023). Somatostatin Neurons from Periaqueductal Gray to Medulla Facilitate Neuropathic Pain in Male Mice. The Journal of Pain, 24(6), 1020–1029. https://doi.org/10.1016/j.jpain.2023.01.002

Zhao, Z., Chen, Z., Xiang, X., Hu, M., Xie, H., Jia, X., Cai, F., Cui, Y., Chen, Z., Qian, L., Liu, J., Shang, C., Yang, Y., Ni, X., Sun, W., Hu, J., Cao, P., Li, H., & Shen, W. L. (2019). Zona incerta GABAergic neurons integrate prey-related

sensory signals and induce an appetitive drive to promote hunting. Nature Neuroscience, 22(6), 921–932. https://doi.org/10.1038/s41593-019-0404-5

Zhong, C.-C., Xu, Z., Gan, J., Yu, Y.-M., Tang, H.-M., Zhu, Y., Yang, J.-X., Ding, H.-L., & Cao, J.-L. (2024). Acute Ongoing Nociception Delays Recovery of Consciousness from Sevoflurane Anesthesia via a Midbrain Circuit. The Journal of Neuroscience, 44(34), e0740242024. https://doi.org/10.1523/JNEUROSCI.0740-24.2024

Zhong, P., Zhang, Z., Barger, Z., Ma, C., Liu, D., Ding, X., & Dan, Y. (2019). Control of Non-REM Sleep by Midbrain Neurotensinergic Neurons. Neuron, 104(4), 795-809.e6. https://doi.org/10.1016/j.neuron.2019.08.026

Zhu, H., Xiang, H.-C., Li, H.-P., Lin, L.-X., Hu, X.-F., Zhang, H., Meng, W.-Y., Liu, L., Chen, C., Shu, Y., Zhang, R.-Y., Zhang, P., Si, J.-Q., & Li, M. (2019). Inhibition of GABAergic Neurons and Excitation of Glutamatergic Neurons in the Ventrolateral Periaqueductal Gray Participate in Electroacupuncture Analgesia Mediated by Cannabinoid Receptor. Frontiers in Neuroscience, 13, 484. https://doi.org/10.3389/fnins.2019.00484

Zhu, X., Xu, Y., Shen, Z., Zhang, H., Xiao, S., Zhu, Y., Wu, M., Chen, Y., Wu, Z., Xu, Y., He, X., Liu, B., Liu, J., Du, J., Sun, J., Fang, J., & Shao, X. (2022). Rostral Anterior Cingulate Cortex–Ventrolateral Periaqueductal Gray Circuit Underlies Electroacupuncture to Alleviate Hyperalgesia but Not Anxiety-Like Behaviors in Mice With Spared Nerve Injury. Frontiers in Neuroscience, 15. https://doi.org/10.3389/fnins.2021.757628

Zhu, X., Zhang, C., Hu, Y., Wang, Y., Xiao, S., Zhu, Y., Sun, H., Sun, J., Xu, C., Xu, Y., Chen, Y., He, X., Liu, B., Liu, J., Du, J., Liang, Y., Liu, B., Li, X., Jiang, Y., … Fang, J. (2024). Modulation of Comorbid Chronic Neuropathic Pain and Anxiety-Like Behaviors by Glutamatergic Neurons in the Ventrolateral Periaqueductal Gray and the Analgesic and Anxiolytic Effects of Electroacupuncture. eNeuro, 11(8), ENEURO.0454-23.2024. https://doi.org/10.1523/ENEURO.0454-23.2024

Chapter 2: Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature

Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature

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REVIEW

Special Issue: Pain

Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature

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Abstract

The neurotransmitter glycine is an agonist at the strychnine-sensitive glycine receptors. In addition, it has recently been discovered to act at two new receptors, the excitatory glycine receptor and metabotropic glycine receptor. Glycine's neurotransmitter roles have been most extensively investigated in the spinal cord, where it is known to play essential roles in pain, itch, and motor function. In contrast, less is known about supraspinal glycinergic functions, and their contributions to pain circuits are largely unrecognized. As glycinergic neurons are absent from cortical regions, a clearer understanding of how supraspinal glycine modulates pain could reveal new pharmacological targets. This review aims to synthesize the published research on glycine's role in the adult brain, highlighting regions where glycine signaling may modulate pain responses. This was achieved through a scoping review methodology identifying several key regions of supraspinal pain circuitry where glycine signaling is involved. Therefore, this review unveils critical research gaps for supraspinal glycine's potential roles in pain and pain-associated responses, encouraging researchers to consider glycinergic neurotransmission more widely when investigating neural mechanisms of pain.

KEYWORDS
glycine, pain, GlyR, metabotropic glycine receptor, excitatory glycine receptor

1 | INTRODUCTION

1.1 | Overview of the review scope

In the central nervous system (CNS), glycine is best known for its action as a fast inhibitory neurotransmitter acting at inhibitory glycine receptors (GlyR; Lynch, 2004). More recently, glycine has been shown to interact with two new glycine-activated neuronal receptors, the excitatory glycine receptor (eGlyR; Grand et al., 2018; Otsu et al., 2019) and metabotropic glycine receptor (mGlyR; Laboute et al., 2023). Additionally, glycine has long been recognized as a co-agonist with glutamate at traditional N-methyl-D-aspartate receptors

Abbreviations: ACC, anterior cingulate cortex; BLA, basolateral amygdala; BötC, Bötzinger complex; CNS, central nervous system; eGlyR, excitatory glycine receptor; GABA, gamma-aminobutyric acid; GAD65/67, glutamic acid decarboxylase 65/67; GluN3A, 3A subunit of N-methyl-D-aspartate glutamate ionotropic receptor; GlyR, glycine receptor; GlyT2, glycine transporter 2; GPR158, G protein-coupled receptor-158; HPF, hippocampal formation; ISH, in situ hybridization; KO, knockout; LH, lateral hypothalamus; lPBN, lateral parabrachial nucleus; MeSH, medical subject headings; mGlyR, metabotropic glycine receptor; NMDAR, N-methyl-D-aspartate receptor; NTS, nucleus of the solitary tract; PAG, periaqueductal gray; PBN, parabrachial nucleus; PFC, prefrontal cortex; RVM, rostral ventromedial medulla; S1, primary somatosensory cortex; vGAT, vesicular GABA transporter; vlPAG, ventrolateral periaqueductal gray; VIAAT, vesicular inhibitory amino acid transporter.

(NMDARs; Hansen et al., 2017, 2018; Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Stroebel et al., 2021).

Unlike neurons that release gamma-aminobutyric acid (GABA; Tamamaki et al., 2003) or glutamate (Rothstein et al., 1994), glycinergic neurons are concentrated in the spinal cord and hindbrain with increasingly lighter expression moving into cortical regions, although their axonal fibers are widely distributed (Figure 1; Chéry & De Koninck, 1999; Jonas et al., 1998; Keller et al., 2001; Rampon et al., 1996; Zeilhofer et al., 2005). Glycine's restricted distribution and interactions with distinct receptors suggest that it holds a specialized role in neuronal signaling, and from a therapeutic development perspective, could provide a more nuanced modulation of neuronal function with reduced cognitive side effects (Harvey & Yee, 2013; Vandenberg et al., 2014; Zeilhofer et al., 2018). Considering the distribution of glycinergic neurons and their projections in known pain-responsive regions, along with the variety of receptor targets available, we hypothesize that supraspinal glycinergic neurotransmission may play a role in multiple physiological circuits that regulate pain responses, including sensory, cognitive (motivation and mood), autonomic, and homeostatic systems (respiration and arousal).

Here, we used a scoping review method to assess published data on the role of glycine neurotransmission in adult supraspinal brain regions. We focus on glycine modulation of circuits involved in pain sensation and pain responses, and its interactions with the three receptors at which it is the primary agonist (GlyR, eGlyR, and mGlyR). The review provides a summary of the potential regions, circuits, and functional roles for supraspinal glycine signaling, and highlights unexplored possibilities for this neurotransmitter system in shaping higher brain representations of pain and organizing associated responses.

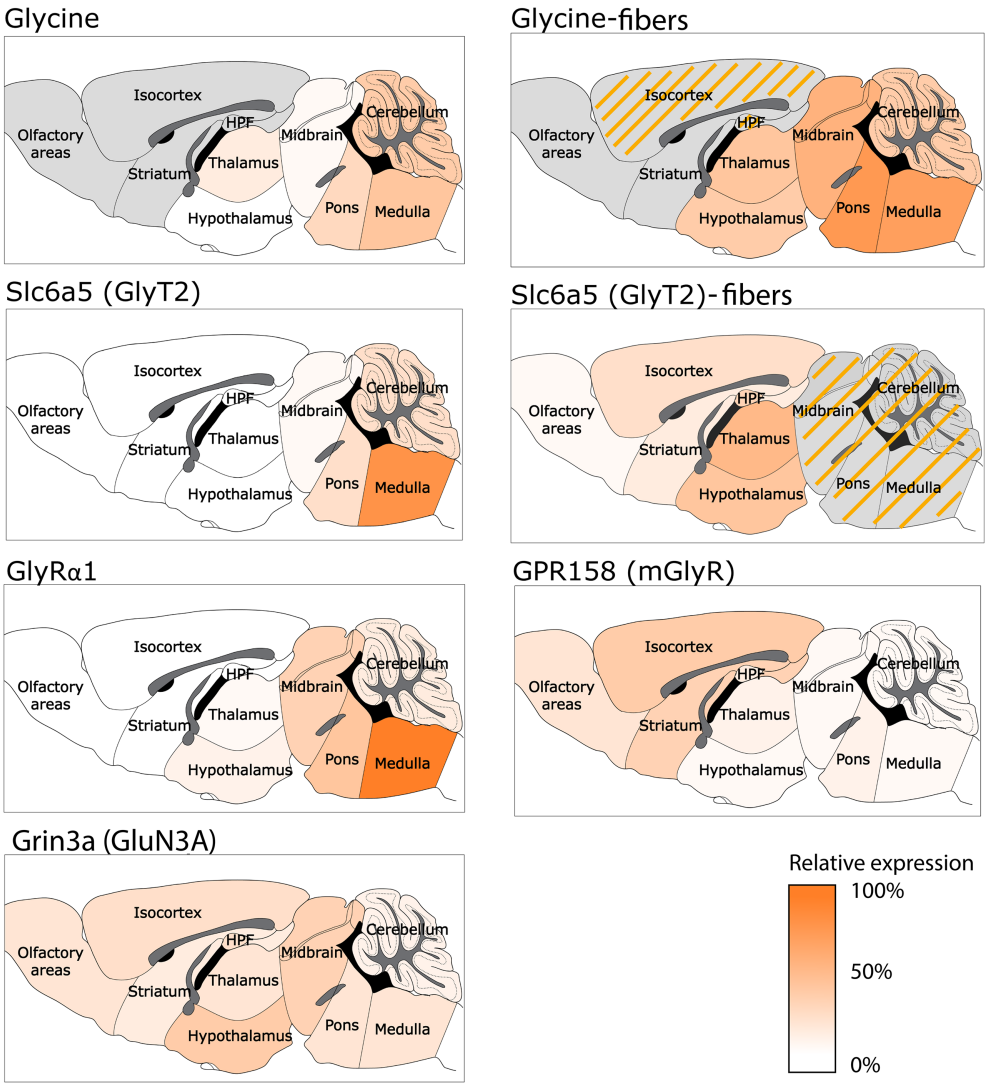

FIGURE 1 Relative abundance of glycine signaling neurons, fibers, and receptors through the rodent brain. Higher relative expression is indicated by more intense color intensity. Regions where glycinergic fibers are reported, but not quantifiable are indicated by orange stripes and areas with no data available are shaded gray. HPF, hippocampal formation (See Table 1 and methods).

TABLE 1 Studies considered in the creation of Figure 1.

Glycine signaling markers	Species	Technique	References
GlyRα1 (RP_050705_02_E12)[a] GlyT2 (*Slc6a5*; RP_050512_02_E05)[a] GPR158 (mGlyR; RP_051121_01_A10)[a] GluN3A (*Grin3a*; RP_050331_03_C02)[a]	Mouse	In situ hybridization	Allen Institute for Brain Science (2004) Lein et al. (2007)
Glycine (glycinergic bodies and fibers)[a]	Mouse	Antibody against glycine protein	Rampon et al. (1996)
Glycine and GlyR	Rat	Antibody against glycine and GlyR protein	van den Pol & Gorcs (1988)
GlyR	Rat	Antibody against GlyR protein	Araki et al. (1988)
GlyR	Human	Antibody against GlyR protein	Baer et al. (2009)
GlyR	Human	Autoradiography using strychnine	Probst et al. (1986)
GlyR subunits	Rat	In situ hybridization	Malosio et al. (1991)
GlyT2 (fiber data only[a])	Mouse	Transgenic mouse	Zeilhofer et al. (2005)
GlyT2	Rat	In situ hybridization	Tanaka and Ezure (2004)
GPR158 (mGlyR)	Mouse	Transgenic mouse	Chang et al. (2023)
Grin3a	Mouse	In situ hybridization	Murillo et al. (2020)

Note: All sources considered reported either mRNA or protein expression in rodent tissue.
[a]Relative expression data available.

1.2 | Glycine is an agonist at three distinct neuronal receptors: GlyR, eGlyR, and mGlyR

Glycine is best known as an agonist at the strychnine-sensitive GlyR, a pentameric chloride channel that is a member of the cys-loop ligand gated ion channel family (Callister & Graham, 2010; Legendre, 2001; Lynch, 2004). As a chloride permeable channel, the mechanism of GlyR inhibition of neurons is similar to $GABA_A$ receptors; however, its kinetics are generally faster, and GlyRs have a distinct set of endogenous and exogenous modulators (Lynch, 2009). A good indicator of GlyR localization is the expression pattern of alpha-1 glycine receptor subunits (GlyRα1; Figure 1), as the latest evidence suggests the predominant subunit composition for GlyRs is 4α:1β subunits, although some controversy remains (Grudzinska et al., 2005; Lynch, 2004; Sato et al., 1991; Zhu & Gouaux, 2021). In both rodents and humans, GlyRs are most strongly expressed in the spinal cord and brainstem, as well as the midbrain, thalamic regions, and hippocampus, with little reported in forebrain areas (Baer et al., 2009; Lynch, 2004; Probst et al., 1986). GlyRs are found in both the pre- and postsynaptic terminal, as well as extrasynaptically (Baer et al., 2009; Lynch, 2004; Sato et al., 1991; van den Pol & Gorcs, 1988; Zeilhofer et al., 2005).

NMDARs are tetrameric receptors constructed from a combination of glutamate-binding GluN2-subunits and glycine-binding GluN1, or less frequently GluN3 subunits; however, eGlyR is a receptor formed exclusively by GluN1 and GluN3A subunits (Bossi et al., 2023; Hansen et al., 2017; Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). As a result, eGlyR is a cation-permeable receptor exclusively activated by glycine. Thus far, functional eGlyRs have only been found in three adult mammalian brain regions which all express high levels of GluN3A (Bossi et al., 2022; Murillo et al., 2020; Otsu et al., 2019; Wong et al., 2002). In the medial habenula, eGlyR modulates place aversion (Otsu et al., 2019); in the basolateral amygdala, it plays a role in the formation of fear memories; and in the neocortex, it is selectively expressed in somatostatin-positive interneurons (Bossi et al., 2022). Interestingly, all three of these regions lack glycinergic inputs and glial-mediated fluctuations in extracellular glycine are thought to adjust the tonic neuronal excitability of the cells in which it is expressed (Otsu et al., 2019). Other regions including the hypothalamus and midbrain have been reported to have high GluN3A expression in both rodent and primate, suggesting that eGlyR or GluN3-containing NMDARs could be expressed (Henson et al., 2008; Mueller & Meador-Woodruff, 2005; Murillo et al., 2020; Wong et al., 2002). Currently, the understanding of how and when eGlyR contributes to neuronal signaling and behavior is hindered by a lack of selective pharmacological agents or genetic tools, in particular ones capable of isolating eGlyR function from GluN3 containing NMDARs.

In 2023, glycine was shown to be the agonist of the orphan receptor GPR158 (G-protein-coupled receptor 158; Laboute et al., 2023) and it was redesignated as mGlyR. mGlyR preferentially binds to

the regulator of G-protein signaling 7 (Orlandi et al., 2012, 2015) and is strongly expressed throughout the rodent brain, particularly the neocortex, striatum, and olfactory regions (Chang et al., 2023). mGlyR is upregulated in the prefrontal cortex (PFC) of people with major depression, and in mice exposed to an unpredictable chronic mild stress, or physical restraint stress. Furthermore, its overexpression in layers 2/3 in the medial PFC induced a depressive-like phenotype in mice (Sutton et al., 2018). Finally, mGlyR knockout (KO) mice seem to have learning deficits as they perform badly in the Morris water maze and novel object recognition tasks (Çetereisi et al., 2019; Khrimian et al., 2017). Together, these studies strongly suggest that mGlyR in the PFC modulates emotional states and influences memory, but how mGlyR achieves this and what its contribution to signaling in other brain regions and circuits remains to be investigated.

An overview of the rodent distribution of glycine, GlyT2, and markers of the three glycine receptors (GlyR, eGlyR, and mGlyR) is listed in Table 1 and is shown in Figure 1.

1.3 | The neurotransmitter glycine is often co-released with GABA

Glycinergic neurons are defined by their expression of the membrane glycine transporter 2 (GlyT2) in combination with the vesicular inhibitory amino acid transporter (VIAAT; Zafra et al., 2016; Zeilhofer et al., 2005) which work together to accumulate the high concentration of glycine needed to package it into synaptic vesicles (Aubrey et al., 2007; Rousseau et al., 2008; Vaaga et al., 2014). As expression of GlyT2 and VIAAT is tightly correlated, GlyT2 is considered a reliable marker of glycinergic neurons (Poyatos et al., 1997).

In addition to glycine, VIAAT also concentrates GABA into synaptic vesicles and for this reason is often called the vesicular GABA transporter (vGAT; McIntire et al., 1997). As such, VIAAT-expressing neurons that contain GlyT2 and a GABA-synthesizing enzyme (Glutamic acid decarboxylase 65/67, GAD65/67) can co-accumulate and co-release glycine and GABA from the same synaptic vesicle (Aubrey et al., 2007; Jonas et al., 1998; Vaaga et al., 2014). Indeed, GABA and glycine are usually co-released in adult mammalian neurons, although there are a few exceptions (Ezure, Tanaka, & Kondo, 2003; Miranda et al., 2022).

Importantly, the quality of signaling at synapses where glycine and GABA are co-released is ultimately determined by the cohort of postsynaptic receptors expressed in combination with the balance of glycine/GABA released from the presynaptic terminal (Apostolides & Trussell, 2013; Aubrey & Supplisson, 2018; Dugué et al., 2005; Dumontier et al., 2023). For example, cerebellar Golgi cells that corelease glycine/GABA signal through postsynaptic $GABA_A$ receptors at their granule cell terminations, but GlyR at their unipolar brush cell terminations (Dugué et al., 2005) and competition for vesicular uptake at GABA/glycine co-releasing synapses can reduce net inhibitory strength compared to synapses that signal with GABA alone (Dumontier et al., 2023). Additionally, glycine can interact with extra- or presynaptic receptors instead of postsynaptic ones (Chéry & De Koninck, 1999; Choi et al., 2013; Lee et al., 2009; Mukhtarov et al., 2005). Thus, glycine is often co-released with GABA and can employ a variety of signaling strategies to alter neuronal function.

1.4 | The neurocircuitry of pain

The physiological role of pain is to prevent or limit tissue damage now and in the future. For this, pain engages the sensory nociceptive circuits involved in the transmission of noxious information, as well as those required to integrate the affective (emotional), motivational, memory-related, and learning-centered components of pain (Price, 2000). Pain also triggers autonomic pain-associated responses, such as those responsible for coordinating arousal and cardiovascular function (Hsueh et al., 2023; Jafari et al., 2017; Liu et al., 2022).

The basic circuits governing nociception are well described (Basbaum et al., 2009; Denk et al., 2014). Briefly, the ascending pain pathway sends noxious information from the periphery to the spinal cord and then higher brain regions including the parabrachial nucleus (PBN), the ventral posterolateral nucleus of the thalamus, and the primary somatosensory cortex (S1), where the signal is perceived as "pain." The nucleus of the solitary tract (NTS) and trigeminal nuclei receive sensory nociceptive information particularly related to noxious stimuli from the viscera and face, respectively (Al-Khater & Todd, 2009; Chiang et al., 2019; Wang et al., 2022). Top-down modulation of nociception occurs via the descending pain modulatory pathways which alter the strength of incoming noxious signals at the level of the spinal cord. The best studied of these originates in the midbrain periaqueductal gray (PAG) and passes through the rostral ventromedial medulla (RVM) before terminating in the dorsal horn of the spinal cord (Figure 2; Lau & Vaughan, 2014).

The higher order areas associated with the emotional and learning aspects of pain are identified from brain imaging studies of both humans and rodents. These include the amygdala and hippocampus, known for their roles in fear, anxiety, and memory; cognitive areas such as the insular, cingulate, and prefrontal cortex, which are involved in pain evaluation and expectancy; and autonomic and homeostatic areas involved in cardiovascular responses and arousal, such as the hypothalamus, Bötzinger complex (BötC), and cerebellum (Figure 2; Da Silva & Seminowicz, 2019; Kuner & Kuner, 2021; Leknes & Tracey, 2008; Morton et al., 2016). Here, we identified and evaluated papers using a scoping review method that provides evidence for glycinergic signaling in regions related to nociception, affective pain components, and other pain-associated responses (Figure 2).

2 | AIMS AND METHODS

We employ a scoping review method to identify published data providing experimental evidence that glycinergic signaling has a role in

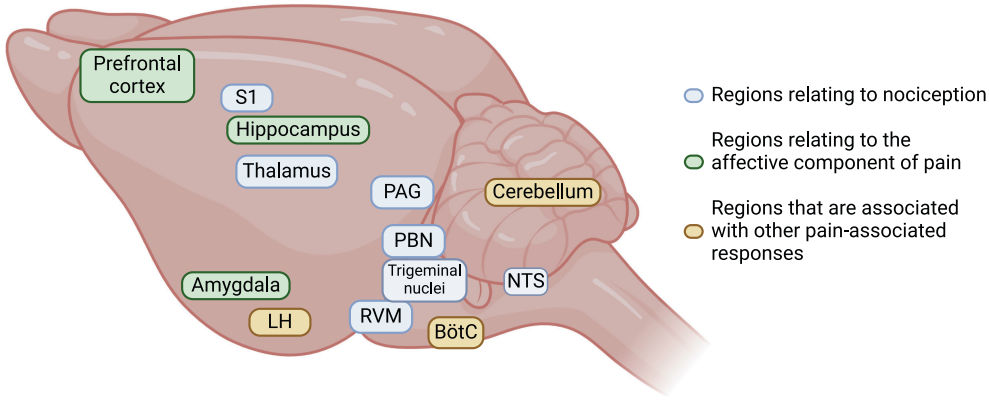

FIGURE 2 Major supraspinal brain regions that modulate pain. Incoming noxious signal from the periphery enters the CNS at the level of the spinal cord before being transmitted to the cortex by the ascending and modulated by descending pain pathways (blue). How this sensory signal is integrated to shape the pain experience and coordinate dynamic responses is less clear, but the mechanisms are key to understanding the distress, dysfunction, and disability that occurs in patients experiencing chronic pain. In addition to nociceptive pathways (blue), we also consider the evidence for glycine signaling in regions that are strongly implicated in the affective components of pain (green), and other pain-associated responses (e.g., autonomic or homeostatic; orange). BötC, Bötzinger complex; LH, Lateral hypothalamus; NTS, Nucleus of the solitary tract; PAG, Periaqueductal gray; PBN, Parabrachial nucleus; RVM, Rostral ventromedial medulla; S1, Primary somatosensory cortex. Image created with BioRender.

the supraspinal modulation of nociception and pain responses in the adult brain. It considers glycine interactions with the three neuronal receptors at which it is the primary agonist: GlyR, eGlyR, and mGlyR.

Using this method, this review aims to (1) synthesize what is known about supraspinal glycinergic signaling in the adult brain; (2) highlight its roles in regions where it may shape higher brain representations of pain, or organize pain responses, or both; and (3) highlight critical research gaps to be explored.

2.1 | Scoping review method

2.1.1 | Search

An updated scoping review search was conducted, with initial searches conducted between June and November 2023 (see Figure 3 for the PRISMA flow diagram of review method). Searches had no date limits for both published and unpublished data (gray literature) within Medline (from 1946 onwards) and Embase (from 1974 onwards) databases (via OVID). A combination of medical subject headings (MeSH) and multipurpose search terms was used in relation to glycine, neurotransmission (or synaptic transmission), brain, and neurons (or nerve cells). In total, 790 references were identified in the initial search. Complete search parameters are presented in Data S1.

2.1.2 | Screening and data extraction

The searches on the different databases were combined in OVID and de-duplicated (672 references remained). Following this, reference titles and grouped references were screened based on the brain region or the physiological outcome of interest. At this stage, references that did not focus on glycinergic signaling were removed (143 references remained). Common themes that were addressed in the disregarded references were 1. measuring glycine serum levels in cerebrospinal fluid; 2. considering glycine with relation to its co-agonist action with glutamate at NMDARs; and 3. studies focused on glycine's role in spinal cord signaling, metabolism, or in systems outside the brain.

Finally, the abstract and key findings of the remaining papers were screened. Studies were included in this review if they identified a role for glycinergic signaling in a brain region or pathway. During this stage, key findings were extracted and summarized. In addition to the exclusion criteria stated above, studies that identified a negative result for glycinergic transmission (i.e., glycinergic signaling was unaffected/unchanged), or did not provide any new information about glycine signaling were excluded. On the other hand, during this part of the screening process, reference lists of identified articles were used to search for additional relevant publications (67 additional papers were added). In the case where the scoping process identified conference abstracts, the subsequent published article was sought, and if found, replaced the conference data. As a result of this process, 135 references were considered in this review. After grouping them by brain regions, 83 papers were found to investigate glycinergic signaling in brain regions with a link to nociceptive, affective, or other pain-associated responses (Figure 2). Most papers considered glycinergic transmission at GlyR with less than 6% of papers reviewed considering glycine signaling at either eGlyRs or mGlyRs. The 52 papers about glycine in brain regions without links to pain are not discussed in the text but are summarized in Table S1.

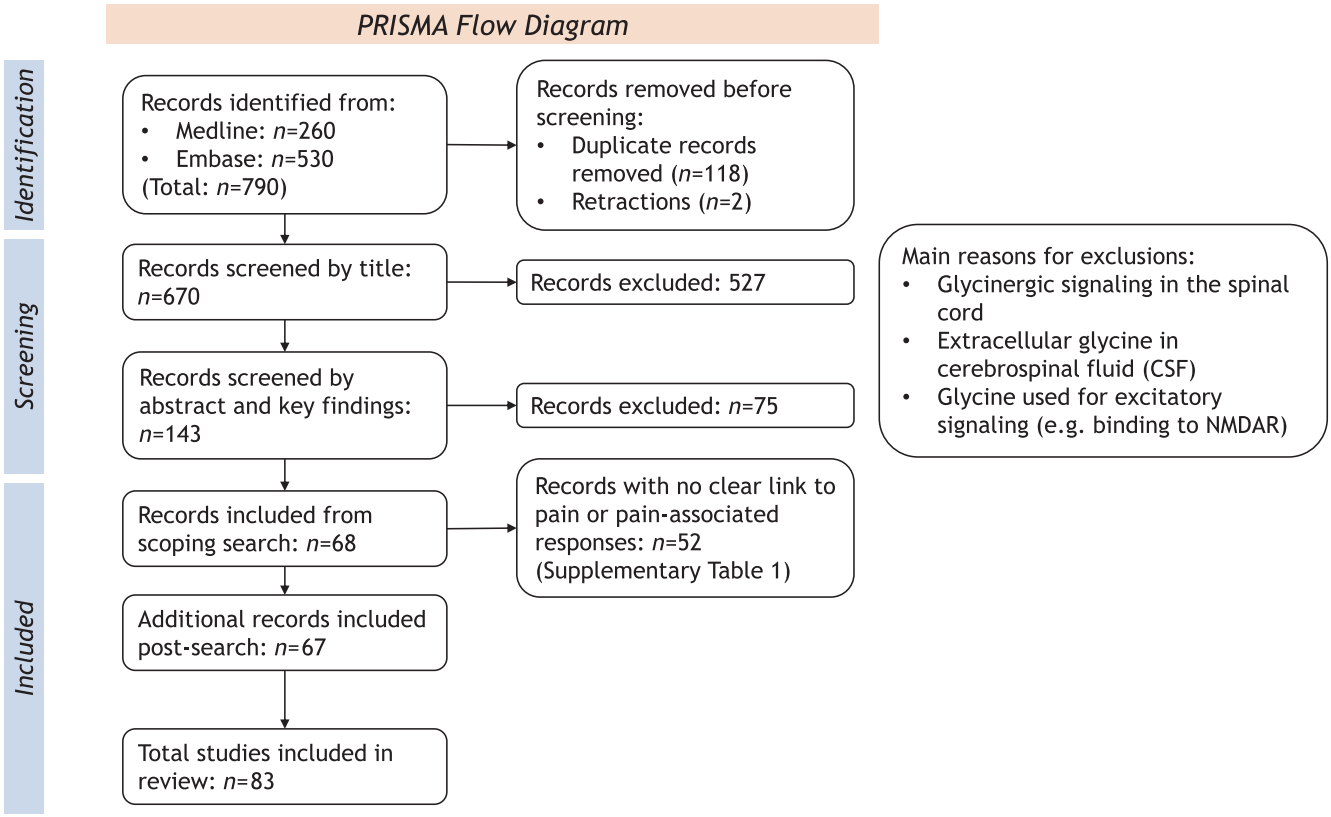

FIGURE 3 PRISMA flow diagram of review method. Papers were identified using a scoping review method using Medical Subject Headings (MeSH) and multipurpose search terms. Then, reference lists of identified articles were used to search for additional relevant publications.

2.2 | Data used to construct relative abundance figure

Several sources reporting glycinergic neurotransmission-related message or protein expression were considered (Table 1; Figure 1; Buccitelli & Selbach, 2020). It should be noted that previous studies have demonstrated post-translational modification of GlyR and GlyT2, but these studies are spinal cord focused (de la Rocha-Muñoz et al., 2019; Moraga-Cid et al., 2020; Zhang et al., 2019). Only expression sources reporting relative expression data were suitable for quantification (*). Raw expression values of mRNA obtained from the Allen Mouse Brain in situ hybridization (ISH) Data database indicate the position of cell bodies that express *Slc6a5* (gene encoding mouse GlyT2), GlyRα1, GPR158, and *Grin3a* (gene encoding mouse GluN3A). These were converted to a 20-point scale of expression for each major brain region. The fluorescent intensity values reported for GlyT2 protein in neuronal fibers, and glycine in cell bodies (Rampon et al., 1996; Zeilhofer et al., 2005), were first converted to a five-point scale (0: no signal - ++++: high signal) which was averaged across subregions before being normalized to a 20-point scale for each major brain region. Reports of very low (e.g., +/−) expression levels were assigned to zero on this scale. Then, the 20-point scale values were converted to a % opacity for shading in Figure 1. Orange stripes are used to mark regions where glycine cells or fibers are reported but no relative expression values are available, and gray marks regions with no available data.

3 | EVIDENCE FOR GLYCINE MODULATION OF SUPRASPINAL PAIN CIRCUITS

The evidence highlighted in the review process was generated primarily from fundamental studies in naïve rodent models. In cases where evidence presented was derived from humans or other species, or where a specific preclinical model of a disease state was investigated (e.g., a persistent pain state), it has been clearly indicated.

3.1 | Preliminary evidence that glycinergic transmission and GlyRs have a role in sensory and pain circuits

A role for GlyR and glycinergic neurotransmission in respiratory, motor, and sensory systems was first highlighted by the symptoms of poisoning with the selective GlyR-antagonist strychnine. In humans, strychnine poisoning results in muscle rigidity and facial muscle spasms, causing a characteristic grin ("risus sardonicus"), and suppression of respiratory centers leading to asphyxia, cardiac arrest, and death (Sully et al., 2001; Yamarick et al., 1992). In rodents, administration of increasing doses of strychnine (i.t.) first causes an increased responsiveness to tactile stimuli, followed by muscle jerks and eventual death at high doses (Yaksh, 1989). These findings are

reinforced by studies in genetically modified animals. Glycine transporter KO animals displayed major deficits in respiration and muscle control and died postnatally (Gomeza, Hulsmann, et al., 2003; Gomeza, Ohno, et al., 2003; Graham et al., 2006). In conditional KOs, where spinal glycine neurons are selectively ablated, long-lasting thermal and mechanical hypersensitivity, as well as excessive itch behaviors are observed (Foster et al., 2015), and in animals where midbrain glycine neurons are selectively targeted, their activation and inhibition bidirectionally alter noxious thermal response thresholds (Assareh et al., 2023).

In humans, mutations in key proteins of the glycinergic system result in a rare genetic disorder called hyperekplexia (or startle disease; Andermann et al., 1980; Graham et al., 2006). Hyperekplexia is characterized by an abnormal startle reflex and muscle stiffness in response to unexpected auditory, mechanical, or tactile stimuli (Tijssen et al., 1997; Vuilleumier et al., 2013). Interestingly, hyperekplexia patients also experience hyperalgesia, with lower pain thresholds when compared to healthy controls in sensory behavioral tests such as pressure pain detection, ankle electrical stimulation, and ice water hand submersion (Vuilleumier et al., 2018). Accordingly, similar auditory startle responses and altered sensation can be observed in transgenic mouse models with GlyR mutations (Findlay et al., 2003; Graham et al., 2006; Groemer et al., 2022).

Together, these data provide compelling foundational evidence that glycinergic transmission and GlyR play important roles in sensory and pain circuits, particularly in the spinal cord and midbrain. However, they do not reveal how glycinergic neurons contribute to signaling in different brain regions nor give insight into the potential roles of eGlyR and mGlyR.

3.2 | Supraspinal glycinergic neurotransmission: Nociceptive brain regions

3.2.1 | The ascending pain pathway

Within the ascending pain pathway, only the PBN has strong evidence for a glycine role (Araki et al., 1988; Rampon et al., 1996; Zeilhofer et al., 2005). The only other notable, but isolated, finding is that a light density of glycinergic fibers is reported in the ventral posterior and lateral nucleus of the thalamus as well as layer VI of the S1 (Zeilhofer et al., 2005).

Parabrachial nucleus

The pontine PBN receives sensory information from the spinal cord and has extensive outputs across the brain. As such, it is considered a hub for integrating sensory information and orchestrating appropriate responses to pain and other stressors (Barik et al., 2018; Basbaum et al., 2009; Palmiter, 2018). Projections between the lateral PBN (lPBN) and the amygdala are important for the affective and particularly the aversive components of pain (Chiang et al., 2020; Deng et al., 2020; Li & Sheets, 2020; Raver et al., 2020), and projections to the hypothalamus are involved in organizing arousal levels, feeding suppression, and cardiovascular regulation (Chiang et al., 2020; Liang et al., 2016; Phua et al., 2021). Finally, the lPBN directly interacts with both interneurons and projections neurons in the RVM and lateral PAG, suggesting it may interact with descending pain pathways (Chen et al., 2017; Chiang et al., 2020; Roeder et al., 2016). The lPBN is thought to play a role in the development and maintenance of neuropathic pain as lPBN glutamatergic neurons are sensitized following nerve ligation and their optogenetic stimulation leads to allodynia (Sun et al., 2020).

Evidence from multiple sources suggests that sparse glycinergic cell bodies and a high density of glycinergic axonal fibers are found in the lPBN (Herbert et al., 2000; Rampon et al., 1996; Tanaka & Ezure, 2004; Zeilhofer et al., 2005) and that GlyR is expressed throughout the lPBN and Kölliker-Fuse nucleus, with the notable exception of the external division of the lPBN (Araki et al., 1988; Herbert et al., 2000; Probst et al., 1986). Gene expression for GluN3A is detected in the midbrain, pons, and medulla, making eGlyR a possibility, and mGlyR expression data are yet to be determined (Obolenskaya et al., 2021). Some of the GlyT2-positive axons in the lPBN seem to originate from neurons in the superficial dorsal horn of the spinal cord (Wang et al., 2001); however, clear evidence of synaptic glycine transmission, or a direct demonstration that glycine alters neuronal activity is lacking.

Given the lPBN has well established roles in pain signaling, and that glycinergic neurons, axons, and receptors are all present, there is a reasonable probability that glycinergic signaling is important in the lPBN under some circumstances. A first step to better understand its potential roles is to construct complete input and output maps for glycinergic neurons to determine how glycinergic signaling fits into the lPBN circuit.

Trigeminal nuclei

The trigeminal nuclei (comprised of the mesencephalic nucleus, motor nucleus, principal sensory nucleus, and spinal nucleus) extend from the lateral medulla down to the C3 vertebra (Patel et al., 2024). The spinal trigeminal nucleus, specifically the pars caudalis, carries sensory and nociceptive information from the orofacial region to the ventral posterior medial nucleus of the thalamus and the PBN. The trigeminal nucleus has been shown to be activated by noxious stimuli in animal studies (Abe et al., 2013; Lee et al., 2010) and its damage or dysfunction is implicated in various chronic orofacial pain syndromes in humans (Burstein et al., 2000; Fitzek et al., 2001; Sessle, 2006; Wilcox et al., 2015).

Moderate numbers of glycinergic neurons are present in the spinal trigeminal nucleus (some of which corelease GABA), along with many glycinergic fibers (Avendaño et al., 2005; Rampon et al., 1996). Synaptic GlyR *currents have been recorded and* protein for GluN3A and mGlyR is found (Chang et al., 2023; Wong et al., 2002). Inhibitory circuits involving GABAergic and glycinergic interneurons are proposed to modulate neuronal responses in the trigeminal

nuclei and one report suggests that glycine affects primary sensory afferents by interacting with presynaptic homomeric α3-GlyR (Bae et al., 2016).

A few papers provide indirect evidence that glycine signaling in the spinal trigeminal nucleus is capable of modulating nociception in rodents. First, activation of the spinal trigeminal nucleus in response to meningeal stimulation of C-fibers is enhanced by strychnine (Melin et al., 2017). Second, the activation of *spinal trigeminal neurons by noxious stimulations of the face (capsaicin cream on the whisker pad or constriction of the infraorbital nerve) is inhibited by the engagement of excitatory cortical inputs, a phenomenon prevented by strychnine* (Malmierca et al., 2012). Finally, a β adrenoceptor antagonist, esmolol, that has analgesic actions in humans, enhances glycinergic transmission in the trigeminal nucleus, and trigeminal responses to innocuous stimuli are facilitated by strychnine (Ressot et al., 2001; Yasui et al., 2011). *Together, these studies indicate that glycinergic neurons in the spinal trigeminal nucleus are likely interneurons that act on GlyRs to dampen down nociceptive signals, take part in descending pain modulation, and could contribute to the development of allodynia*.

Nucleus of the solitary tract

The NTS is a medullary region best known for its roles in taste, cardiorespiratory function, and visceral sensation. It integrates both visceral and somatosensation through its inputs from the spinal cord and vagus nerve (Beckers et al., 2022; Boscan et al., 2002; Holt, 2022). In addition, it is strongly linked to descending pain pathways through its extensive connections to the PBN, RVM, and PAG (Holt, 2022; Rinaman, 2010).

Consistent with its direct sensory inputs, both noxious visceral and cutaneous stimuli enhance the activity of neurons in the NTS (Hammond et al., 1992; Pinto et al., 2007). Additionally, responses to a variety of noxious stimuli are reduced when the NTS is electrically stimulated, a phenomenon that is prevented by systemic opioids (Lewis et al., 1987). The proposal that increases in NTS activity are analgesic is directly supported by a study that selectively activated Pro-opiomelanocortin expressing NTS neurons to inhibit acute noxious thermal, mechanical, and persistent inflammatory nociceptive responses and produce transient cardiorespiratory depression. In contrast, inhibition of Pro-opiomelanocortin neurons did not alter nociception but was able to modulate stress-induced analgesia (Cerritelli et al., 2016; Patra et al., 2023). Together, these data demonstrate that NTS neurons respond to nociceptive stimuli but are unlikely to contribute to setting cutaneous nociceptive thresholds and suggest they contribute to organizing behavioral responses to stressors.

A small number of glycinergic neurons as well as moderate glycine fiber densities are reported in the NTS, particularly in the lateral part (Batten et al., 2010; Dufour et al., 2010; Rampon et al., 1996; Zeilhofer et al., 2005). GlyRs, however, are strongly expressed throughout the NTS, with immunohistochemistry and ultrastructure studies demonstrating that GlyR-expressing postsynaptic terminals are opposed to presynaptic terminals that co-package glycine with GABA. Additionally, GlyRs are found in neuronal membranes outside of synapses (Baer et al., 2009; Probst et al., 1986) and mRNA suggests mGlyR and GluN3A are expressed in the NTS (Figure 1).

Microinjection of glycine or strychnine into the NTS affects cardiovascular and respiratory activity, indicating that glycine and GlyRs can alter NTS circuits and function (Cinelli et al., 2016; Kubo & Kihara, 1987; Talman & Robertson, 1989). However, despite its connection within the ascending pain circuitry, only one paper identified provides clear evidence that NTS-glycine signaling is directly involved in nociception. Thek et al. (2019) show that the majority of somatostatin-positive NTS neurons are inhibitory interneurons that release both glycine and GABA and make extensive connections with their neighboring neurons. About half of somatostatin-positive NTS neurons receive direct sensory input from the solitary tract and their activation gates viscero-sensory signals in the NTS, effectively isolating these neurons from their sensory input (Ezure & Tanaka, 2004; Thek et al., 2019; Tsai & Davenport, 2014).

In summary, there is good evidence that glycine signaling at GlyRs helps regulate NTS excitability and function. However, there is much to learn about its role in NTS pain modulation and coordination. For example, how, when, and where glycine signaling occurs, where glycine inputs originate from, and if functional eGlyR and mGlyR are expressed. One intriguing possibility, supported by a study investigating the effect of chronic intermittent hypoxia on NTS neurons, is that NTS neurons that normally receive GABA-dominant inputs can switch to a mixed GABA/glycine phenotype following a physiologic challenge which causes an upregulation of GlyR trafficking and expression at the synapse (Jia et al., 2022).

3.2.2 | The descending pain pathway

The activity of the descending pain pathway directly controls nociceptive inputs at the level of the spinal cord such that stimulation of the ventrolateral region of the PAG (vlPAG), or its activation by microinjections of opioids, can profoundly reduce nociceptive signals in the spinal cord and prevent pain sensation (Bagley & Ingram, 2020; Keay & Bandler, 2008; Lau & Vaughan, 2014).

Periaqueductal gray

The midbrain PAG is a primary control center of descending pain modulation pathways and is critically involved in integrating sensory pain processing, behavioral responses to threats, and autonomic function. It is characteristically divided into functional columns by which the dorsomedial and lateral columns are associated with hypertension, tachycardia, and non-opioid-mediated analgesia; while the ventrolateral column is involved with hypotension, bradycardia, and opioid-mediated analgesia (Bandler & Shipley, 1994; Keay & Bandler, 2008).

Glycinergic cell bodies and fibers are found in the PAG (Rampon et al., 1996; Zeilhofer et al., 2005), with glycinergic neurons concentrated in the vlPAG. This distribution has been confirmed with spatially resolved single-cell transcriptomics and immunolabeling (Tanaka & Ezure, 2004; Uchida et al., 2021; Vaughn et al., 2022).

In contrast, only glycinergic fibers are present in the dorsomedial and lateral columns of the PAG (Keay & Bandler, 2015; Rampon et al., 1996; Zeilhofer et al., 2005). In terms of receptors, there is consistent evidence that GlyRs (α1-3 and β subunits) are present both pre- and postsynaptically in the PAG of both rodents and humans (Araki et al., 1988; Baer et al., 2009; Choi et al., 2013; Malosio et al., 1991; Probst et al., 1986; van den Pol & Gorcs, 1988). Accordingly, strychnine is often used to inhibit locally evoked GlyR-mediated synaptic currents in electrophysiological experiments of the region (Lau et al., 2020; Tonsfeldt et al., 2016; Winters et al., 2022). Preliminary gene expression evidence suggests that GluN3A is highly expressed, and mGlyR is also present (Figure 1), although their distribution throughout the PAG columns has not been resolved and their function is unknown.

Microinjection and microdialysis studies were the first to suggest that glycine signaling in the PAG modulates nociceptive and affective functions. Microinjection of glycine targeted to the dorsal PAG of rats resulted in anxiogenic-like effects in the elevated plus maze test (Schmitt et al., 1995) whereas antagonists for the glycine site of the NMDAR were anxiolytic (Matheus et al., 1994). Glycine microinjection directed to the vlPAG altered heat-induced pain responses and affected the firing rate of neurons in the RVM, indicating modulation of the descending pain pathway (Palazzo et al., 2009). The responses likely involve both GlyRs and NMDARs as they were differentially affected by high or low concentrations of glycine and antagonists against GlyRs or the NMDARs. Finally, endogenous glycine levels are reduced in the PAG following an inflammatory nociceptive stimulus (Maione et al., 2000).

The clearest evidence that glycine neurons in the PAG modulate nociception comes from a chemogenetic study in GlyT2::Cre mice where activation and inhibition of glycinergic PAG neurons bidirectionally modulated mechanical and thermal nociceptive thresholds, such that activation was pronociceptive and inhibition was anti-nociceptive (Assareh et al., 2023). In addition, activation of glycinergic PAG neurons increased locomotion and jumping behaviors. Taken together with similar nociception data generated in vGAT::Cre mice where GABA and glycine-releasing neurons in the vlPAG were simultaneously chemogenetically manipulated (Samineni et al., 2017, 2019), these data provide direct evidence that glycinergic neurons in the vlPAG modulate nociception and suggest a role for them in associated behaviors. Conventional understanding of vlPAG circuity suggests that this modulation is mediated by the descending pain pathway, although this remains to be demonstrated definitively. Interestingly, anterograde and retrograde circuit tracing studies indicate that glycinergic PAG neurons project to the dorsal raphe, locus coeruleus, and the paraventricular nucleus of the hypothalamus (Rampon et al., 1999; Varga et al., 2019), raising the possibility that these neurons could contribute to the PAG's larger integrative role of organizing affective and threat responses. Expanded behavioral experiments while selectively engaging glycinergic PAG neurons are crucial not only for discovering how these neurons affect other PAG-mediated behaviors but also for advancing our understanding of PAG circuitry and the mechanisms underlying how it dynamically shapes pain responses.

Together these data show that glycine signaling in the PAG has a role in nociception, particularly glycinergic neurons in the vlPAG. It may also contribute to affective, defensive, or arousal behaviors through their projections to other nuclei. GlyRs in the PAG are suggested to affect the region's excitability, and the expression of eGlyR and mGlyR is possible, but unverified.

The ventromedial medulla

The ventromedial medulla, particularly the RVM (includes the raphe magnus and gigantocellular reticular nucleus alpha), is core to descending pain modulation (Bagley & Ingram, 2020; Basbaum & Fields, 1984; Lau & Vaughan, 2014) and also contributes to defensive responses by altering autonomic and motor function, including sleep/arousal (Mason, 2005; Morales et al., 2006).

Both glycinergic neurons and extensive glycinergic fibers are found throughout the RVM, particularly in the caudal medulla (Rampon et al., 1996; Tanaka & Ezure, 2004; Zeilhofer et al., 2005). In addition, gene expression for GlyR (α1/2 and β subunits) along with mGlyR and GluN3A is reported (Figure 1), suggesting that synaptically released glycine could act through GlyR, mGlyR, or potentially eGlyR (Araki et al., 1988; Baer et al., 2009; Malosio et al., 1991; Probst et al., 1986; Rampon et al., 1996; Zeilhofer et al., 2005). Behavioral evidence supports a role for glycine in pain modulation, particularly via descending pain pathways. Glycine levels in the RVM are reduced in mice with chronic muscle pain (Radhakrishnan & Sluka, 2009) and anatomical/electrophysiological studies show that GlyT2-positive neurons in the rostral and caudal ventromedial medulla send projections that synapse directly onto a variety of neurons in the superficial dorsal horn of the spinal cord, where they release glycine along with GABA (Hossaini et al., 2012; Otsu & Aubrey, 2022). Finally, RVM projection neurons are reported to receive glycinergic inputs (Finnegan et al., 2004), suggesting that glycine modulates both local neuronal activity as well as being an important component of the descending RVM projection to the dorsal horn of the spinal cord.

In addition to nociception, optogenetic activation of glycinergic neurons located in the nearby lateral paragigantocellular nucleus promotes behavioral arrest and is associated with locomotion and defensive behaviors (Capelli et al., 2017). However, whether glycinergic locomotor neurons participate in coordinating fight and flight/defensive responses in response to pain is unknown.

3.3 | Supraspinal glycinergic neurotransmission: Affective brain regions

The overall physiological role of pain is to prevent or minimize tissue damage in response to environmental dangers and then to avoid them in the future. As a result, appropriate responses to pain require the engagement of affective circuits which are involved in motivating behaviors that avoid, reduce, or escape its presence. Initially, pain provokes an unpleasant feeling and emotions such as fear and

increased arousal. Then, future proofing against pain involves a complex integration of sensory and affective information from multiple anatomically interconnected structures to remember, avoid, and minimize future pain (Price, 2000). Accordingly, the development of chronic pain is linked to affective impairments (such as increased depression and anxiety), memory issues, and cognitive decline (Rouch et al., 2021; Sheng et al., 2017).

Prefrontal cortex

The PFC is responsible for executive functions such as planning, problem-solving, decision-making, and social control. It is also important in pain processing, particularly for motivational and cognitive behaviors in response to pain. The PFC encompasses the anterior portion of the frontal lobe and can be divided into two broad regions, the lateral and medial PFC. Consistent with the PFC's role in the modulation of pain affect, it has strong connectivity to multiple parts of the pain-responsive network including the anterior thalamus, basolateral amygdala (BLA), insula, and PAG (Mathiasen et al., 2020; McGarry & Carter, 2017; Ong et al., 2019). A region of the medial PFC (mPFC) that has been critically linked to affective pain processing and pain avoidance is the anterior portion of the cingulate cortex (ACC; Bliss et al., 2016; Ong et al., 2019; Stegemann et al., 2023; Urien et al., 2018). Indeed, structural and activity changes in the mPFC/ACC are observed following chronic pain induction in both human and rodent brain imaging studies (Apkarian et al., 2005; Peyron et al., 2004; Stegemann et al., 2023). Moreover, through a process of disinhibition, hyperactivity of the ACC is thought to play a fundamental role in increased pain network activity and anxio-depressive comorbidities associated with chronic pain (Blom et al., 2014; Sellmeijer et al., 2018).

There are no glycinergic neuronal soma present in the PFC; however, some GlyT2-positive projections are reported in the cingulate cortex (Rampon et al., 1996; Zeilhofer et al., 2005), and GlyR, mGlyR, and GluN3A are all expressed in the mPFC/ACC, suggesting glycine might play a role here (Araya et al., 2021; Bossi et al., 2023; Chang et al., 2023; Malosio et al., 1991; Murillo et al., 2020; Wong et al., 2002).

While there is currently no evidence that the eGlyR is expressed in the mPFC, numerous studies have reported functional GlyRs are present and mGlyRs have a clear functional role. GlyRs provide strychnine-sensitive tonic control of neuronal excitability, with exogenous glycine application reducing neuronal firing (Liu et al., 2015; Salling & Harrison, 2014). One study also reports activation of GlyR reduces autotomy in a model of neuropathic pain (Pellicer et al., 2007). However, we are not aware of any further evidence directly linking mPFC GlyRs to pain responses. As outlined in the introduction, the discovery of mGlyRs was facilitated by the observation that this G-protein-coupled receptor is upregulated in the PFC of humans with major depressive disorder, and in mice following exposure to short and prolonged periods of stress (Sutton et al., 2018). Coupled with the finding that its overexpression and KO recapitulate stress/memory-related phenotypes, these data suggest that glycine activation of mGlyRs affects the function of the region. However, it remains to be demonstrated where endogenous glycine comes from in the PFC and determines if PFC-mGlyR alters affective behaviors associated with acute or chronic pain.

Amygdala

The amygdala is important for associative learning and is commonly viewed as a region that links emotional and motivational value to a noxious (or appetitive) stimulus (Veinante et al., 2013). Like the ACC, the amygdala is related to the emotional and affective aspects of pain. The amygdala receives nociceptive information from ascending pain pathways via its connection to the lPBN (Torres-Rodriguez et al., 2024) and interacts with descending pain pathways via its projections from GABAergic neurons in the central nucleus of the amygdala to the PAG, which are important for fear behaviors (Tovote et al., 2016; Winters et al., 2022). Additionally, the amygdala has roles in motivational goal-directed decision-making, such as engaging in behaviors to avoid future harm, that involves projections from the BLA to regions of the basal ganglia (Gupta et al., 2011). Its reciprocal connection to cortical regions including the insular cortex, PFC, and ACC helps link sensation to negative valence (Mercer Lindsay et al., 2021), fear extinction (Cho et al., 2013), and conflict assessment in pain models (Valentinova et al., 2023). The amygdala is thus an important point of convergence for many aspects of the pain experience.

There are no glycinergic neurons in the amygdala; however, the central nucleus receives light glycinergic innervation (Rampon et al., 1996; Zeilhofer et al., 2005) and GlyR subunits are expressed in both the central nucleus of the amygdala and the BLA (Ceder et al., 2023; Danober & Pape, 1998; Delaney et al., 2010; McCracken et al., 2017), with one study demonstrating synaptic GlyR-mediated currents are altered when neuropathic pain develops (Oliva et al., 2022). Preliminary gene expression data suggest that mGlyR is lightly and GluN3A is highly expressed, particularly in pyramidal cells of the BLA where functional eGlyR has been reported (Figure 1; Bossi et al., 2022). As there is no evidence for conventional NMDAR containing the GluN3A subunit in this region, the functional role of eGlyRs in the BLA was investigated using a GluN3A knockdown strategy. A comparison of neuronal properties in wild-type and GluN3A KO animals suggests that eGlyR senses extracellular glycine fluctuations (perhaps released from astrocytes) and has a role in setting the excitability of the pyramidal neurons in the BLA. Furthermore, fear memory consolidation is reduced in GluN3A KO animals, suggesting eGlyR is involved in neuroplasticity at these synapses. Establishing the origin and triggers for extracellular glycine fluctuations is key to understanding the roles of both GlyR and eGlyR in the amygdala. The scarcity of glycinergic axonal fibers and suggestions that glycine levels change in response to astrocytic factors (Bossi et al., 2022; Oliva et al., 2022; Otsu et al., 2019) raise the possibility glycine is a neuromodulator rather than a neurotransmitter here.

Hippocampus

The hippocampus is known for its role in learning and memory. In humans, chronic pain is associated with cognitive disruptions, including in memory and learning, and there is mounting evidence that the hippocampus is involved. For example, the activity and connectivity of the hippocampus is decreased, and gray matter volume is reduced in brain imaging studies of people with various forms of chronic pain compared to controls (Neumann et al., 2023). Additionally, preclinical studies show chronic pain reduces dendritic complexity, aberrant synaptic plasticity, and neurogenesis in the hippocampus (Chen et al., 2014; Mutso et al., 2012; Tajerian et al., 2018). The role of the hippocampus in pain is facilitated by its extensive connectivity to pain-responsive brain areas including the BLA (Pi et al., 2020), thalamus (Aggleton et al., 2010; Mathiasen et al., 2020), and hypothalamus (Bang et al., 2022). However, it has no direct circuit connections with the ascending and descending nociceptive pathways, indicating that its effects are achieved by indirect circuits. Despite this, enhanced thermal and mechanical responses induced in a mouse inflammatory pain model can be reduced by optogenetic activation of ventral CA1 hippocampus projections to the BLA, demonstrating that hippocampal activity is able to influence nociceptive responses (Shao et al., 2023).

There are no glycinergic neurons in the hippocampus, but there is evidence of light glycinergic fibers and glycine-positive terminals (Danglot et al., 2004; Van Den Pol & Gorcs, 1988; Zeilhofer et al., 2005). In contrast, mRNA and immunolabeling suggest that GlyR is strongly expressed throughout the region at both pre- and postsynaptic locations. GlyR expression is most prominent in the dentate gyrus, CA1 and CA3 (Chattipakorn & McMahon, 2002; Danglot et al., 2004; Keck & White, 2009; Lee et al., 2009; Mori et al., 2002; Song et al., 2006) and expressed at soma and synaptic membranes (Muller et al., 2013). *Grin3a* expression is high in the hippocampus during development, and it forms functional eGlyR during this period; however, its expression is suppressed in adult tissue (Grand et al., 2018). mGlyR has been reported to be moderately expressed, particularly in the CA3 region of the hippocampus, but its expression, mechanism of activation, and functional role have not been further explored (Chang et al., 2023).

There is a moderate, but well-replicated body of data investigating the role of GlyR in the hippocampus (reviewed in Keck & White, 2009; Xu & Gong, 2010). These data show that GlyR activity in the hippocampus primarily responds to changes in extracellular glycine levels, which could come about from reverse transport of the astrocytic glycine transporter 1 (Ghirardini et al., 2018; Martina et al., 2005), spillover of glycine from distant synapses, or changes in GlyR modulation by endogenous modulators (Chattipakorn & McMahon, 2003; Song et al., 2006; Zhang & Thio, 2007). Indeed, ex vivo recordings suggest that hippocampal GlyRs are tonically active under normal circumstances and their inhibition by strychnine (or modulation by zinc) alters neuronal excitability and promotes long-term depression in response to a 10-Hz conditioning pulse (Keck et al., 2008). Furthermore, enhancing extracellular glycine with a glycine transporter 1 antagonist activates GlyRs and enhances long-term potentiation induction (Zhang et al., 2008). Despite the strong anatomical evidence for synapses, only one paper has reported the detection of small synaptic GlyR currents in the CA1 region of the hippocampus (Muller et al., 2013). Interestingly, this paper presents immunohistochemistry data that suggest glycine is released with glutamate at synapses containing NMDARs, but this has not been further validated. Together, the evidence suggests that hippocampal GlyRs are tonically active, play a fine-tuning role in hippocampal networks and can influence long-term plastic changes. No study has definitively determined the conditions under which extracellular glycine fluctuations occur or the role of hippocampal GlyRs in memory tasks or pain behaviors but, given its links to long-term affective adaptations to pain (Price, 2000), their role in this highly interconnected region warrants further exploration.

3.4 | Supraspinal glycinergic neurotransmission: Other pain-associated brain regions

Dynamically responding to pain and other stressors requires a constant integration of sensory information with homeostatic and autonomic control centers. For example, in addition to nociceptive and affective engagement, appropriate fight or flight responses require coordination of arousal, motivation, cardiac and respiratory functions. A mechanistic understanding of how cardiac function and breathing influence pain and anxiety has begun to be elucidated (Hsueh et al., 2023; Liu et al., 2022).

Lateral hypothalamus

The lateral hypothalamus (LH) mediates a range of physiological responses including feeding behaviors, motivation, reward, and sleep. In addition, a growing body of evidence indicates that it also has a significant role in pain responses, both for nociception and in coordinating pain-stimulated changes in motivation, feeding, reward, and sleep (Bouâouda & Jha, 2023; Jennings et al., 2015; Petrovich, 2018; Stuber & Wise, 2016). The LH contains many pain-responsive neurons and has ample circuit connections with the ascending and descending pain pathways. This includes connections with the spinal cord, the PBN, the PAG, and the amygdala, suggesting it is part of the pain-responsive network (Behbehani et al., 1988; Burstein et al., 1987; Dafny et al., 1996; Pauli et al., 2022; Weera et al., 2021).

The LH does not contain glycinergic somas but has significant input from glycinergic fibers (Rampon et al., 1996; Zeilhofer et al., 2005). Anatomical evidence shows that some LH synapses express GlyRs (Hondo et al., 2011; Karnani et al., 2011) and GluN3A expression is high, suggesting eGlyR may be present; however, detailed spatial expression data for GluN3A and functional evidence for eGlyR are lacking (Ciabarra et al., 1995). There is currently no evidence of mGlyR's presence or absence in the LH.

A functional role for glycine in the LH has been most convincingly explored in LH orexin-releasing neurons. Orexin neurons are exclusively concentrated in the LH (and adjacent perifornical area)

and project widely throughout the brain. They are famously known for their role in wake/sleep regulation (Yamashita & Yamanaka, 2017). However, they also influence many other functions including pain (Li & de Lecea, 2020). Indeed, administration of orexin into the PAG, RVM, or spinal cord (which all receive orexin neuron inputs) is strongly antinociceptive (Azhdari Zarmehri et al., 2011; Azhdari-Zarmehri et al., 2014; Ho et al., 2011; Kajiyama et al., 2005; Wang et al., 2018a, 2018b; Yamamoto et al., 2003). Electron microscopy and patch clamp recordings suggest orexin neurons receive direct input from glycine-containing terminals and express functional GlyR (Hondo et al., 2011; Karnani et al., 2011). Exogenous glycine reduces orexin neuron activity, but evidence for endogenously released glycine and synaptic glycine currents is missing. Thus, glycine can potentially modulate orexin neurons which would plausibly affect nociceptive signaling.

Indirect evidence suggests that glycine modulation of orexin neurons could contribute to changes in arousal. This is of interest as pain generally promotes arousal and because sleep disorders are a well-known comorbidity in patients with chronic pain. First, peripheral administration of glycine reduces the activity of orexin neurons and increases sleep time during dark periods in rodents (Hondo et al., 2011; Karnani et al., 2011). Second, oral administration of glycine improves sleep quality in humans, correlating with polysomnographic changes (Bannai & Kawai, 2012; Yamadera et al., 2007). Although we could speculate that these data are mediated by glycine's actions in the LH, a more direct demonstration of this is needed to be convincing. In summary, there is light evidence that glycine alters the activity of LH neurons, and if so, its actions could impact pain sensation and/or arousal.

Cerebellum

The cerebellum is a highly organized region primarily known for its role in motor functions like balance, coordination, and voluntary motor control. However, emerging roles in non-motor functions such as cognition and emotion are increasingly being reported (Baumann et al., 2015; Lawrenson et al., 2018; Rudolph et al., 2023; Saab & Willis, 2003). In addition, changes in human cerebellar activities monitored through positron emission tomography or functional magnetic resonance imaging are temporally correlated to painful stimuli, and chemical or electrical stimulation of the cerebellum alters the intensity of nociceptive responses in rodents (Moulton et al., 2010; Saab & Willis, 2002).

In both the cerebellar cortex and nuclei, there is significant expression of glycinergic cell bodies and fibers (Bagnall et al., 2009; Rampon et al., 1996; Simat et al., 2007; Zeilhofer et al., 2005). In addition, gene expression for both mGlyR and GluN3A (molecular and granular cell layer) is present (Chang et al., 2023; Wong et al., 2002).

While the mechanisms underlying these findings remain unclear, direct connections between the cerebellum and several nuclei that modulate pain have been consistently reported. This includes inputs from the pons and hypothalamus, and outputs to the vlPAG, the PBN, and the ventral tegmental area (Koutsikou et al., 2014; Rudolph et al., 2023; Schmahmann, 1996; Vaaga et al., 2020). Notably, the cerebellar fastigial nucleus sends glutamatergic projections to the vlPAG where they synapse with both glutamatergic and GABAergic vlPAG neurons that are thought to be involved in the sensorimotor aspects of fear learning responses (Frontera et al., 2020). The demonstration that stimulation (or disinhibition) of the cerebellar fastigial nucleus reduces responses to a noxious visceral stimulus (Saab & Willis, 2002) suggests its activity can alter nociception perhaps via its interactions with the descending pain pathway. On the other hand, GABAergic Purkinje cells (the main output neuron of the cerebellar cortex) have monosynaptic connections with a subset of neurons that are well known to drive pain-related behaviors (Tachykinin receptor 1-expressing PBN neurons; Barik et al., 2018; Chen et al., 2023). Thus, the cerebellum is well placed to directly respond to and/or influence nociception through its interactions with the ascending and descending pain pathways.

Glycinergic transmission through GlyRs is known to contribute to both cerebellar output and its regulation (Ankri et al., 2015; Bagnall et al., 2009; Uusisaari & Knöpfel, 2010). Cerebellar glycine-GABA releasing neurons are reported in several regions, including interneurons in the granule cell layer of the cerebellar cortex, such as Lugaro/globular cells and Golgi cells (Dugué et al., 2005; Dumoulin et al., 2001; Simat et al., 2007) and some Purkinje cell which projects onto principal neurons in the cerebellar nuclei which have a minor glycinergic component (<3% of the inhibitory current; Husson et al., 2014). By exploiting knowledge about the organization of GABA/glycine-releasing cerebellar neurons, it has been shown that both presynaptic and/or postsynaptic factors shape neurotransmission at their synapses, and that the quality of inhibition at co-releasing synapses is different compared to synapses where only GABA is released (Dugué et al., 2005; Dumontier et al., 2023). Therefore, inhibitory synaptic signaling which is mediated by glycine modulates cerebellar activity. While the activity of the cerebellum clearly influences pain behaviors, whether or not glycine-mediated synaptic signaling here contributes to its pain-relevant functions is currently unknown. Possible roles include coordinating motor responses to pain (e.g., fear-evoked freezing) or cognitive-affective functions (Koutsikou et al., 2014; Rudolph et al., 2023; Vaaga et al., 2020), but this remains to be investigated.

PreBötC and BötC

The preBötC and BötC are essential for organizing the periodic drive for inspiration and active expiration, respectively, and interact to coordinate respiratory patterns (Del Negro et al., 2018; Ezure, Tanaka, & Saito, 2003; Flor et al., 2020; Schreihofer et al., 1999). Pain-induced changes in breathing rate are mediated by neurons in the pre-BötC that receive inputs from a group of opioid receptor mu 1-positive neurons in the central lPBN and activation of this pathway increases breathing rate and pain perception, while its inhibition reduces breathing rates without affecting pain perception (Liu et al., 2022).

Glycinergic neurons and their fibers are concentrated within the preBötC and BötC, with anatomical and functional evidence supporting key roles for GlyR-mediated signaling (Morgado-Valle et al., 2010; Schreihofer et al., 1999). Interestingly, glycinergic neurons that do not corelease GABA are reported (Ezure, Tanaka, & Kondo, 2003), suggesting they may have specialized roles.

Preliminary data indicates that expression of eGlyR and mGlyR is possible (Figure 1).

As glycinergic neurotransmission in the preBötC and BötC is known to alter respiratory patterns and control respiratory phase transition and duration control (Borrus et al., 2020; Ezure, Tanaka, & Saito, 2003; Lieske et al., 2000; Marchenko et al., 2016; Sherman et al., 2015; Smith et al., 2007), and anatomical evidence demonstrates that glycinergic preBötC neurons project throughout the brain, including back to the lPBN and to parts of the thalamus and hypothalamus, a role for glycinergic neurons in pain-induced breathing regulatory circuit seems likely. However, as yet no study has directly investigated their role in the coordination of breathing with other behaviors or emotional states such as pain (Yang & Feldman, 2018).

4 | CONCLUDING REMARKS

We have provided a broad summary of existing evidence of supraspinal glycine signaling in regions known to be involved in modulating pain sensation and pain-associated responses. We found clear evidence that glycine neurotransmission plays a role in nociception and likely modulates affective and other pain-associated responses. However, there is much work needed to understand which neurons and microcircuits are involved, how and when glycine levels fluctuate near glycine receptors, and the mechanisms by which GlyR, eGlyR, and mGlyR contribute to neuronal excitability and function.

It is striking that, in multiple regions covered in this review, glycine receptors are expressed and can alter neuronal function, but it is unclear how and when these receptors are engaged as receptor expression is sometimes uncoupled from the inputs necessary for synaptic glycine release (such as in the hippocampus, amygdala, and medial prefrontal cortex). Glycine's extra-synaptic concentration is determined by a combination of neuronal architecture, synaptic release, and the expression and activity of specific and non-specific uptake mechanisms (Aubrey et al., 2005; Beato et al., 2004; Supplisson & Roux, 2002). As such, possible alternative mechanisms for glycine fluctuations include oscillations in cerebrospinal fluid glycine levels, glial-mediated release, or spill-over from nearby regions (Bossi et al., 2023; Otsu et al., 2019; Xu & Gong, 2010). Despite this lack of clarity, the evidence summarized here suggests that, in regions with and without glycinergic axonal input, the potential for glycine signaling to contribute to higher brain function is noteworthy and should not be overlooked. This may be explained by a lack of published data or reliable experimental tools but may also indicate that extrasynaptic glycine receptors are more important than normally considered, and there are as yet undiscovered ways in which extracellular glycine levels are regulated. This is particularly important considering the new research areas triggered by the discoveries of eGlyR and mGlyR.

The literature covered suggests that building knowledge about supraspinal glycinergic signaling will refine our understanding of pain circuitry and contribute new insights into the mechanisms by which pain is modulated and pain responses organized. Together, this knowledge aids in better explaining clinical pain phenomena and could highlight new therapeutic strategies to precisely modulate specific aspects of pain behavior, particularly when it becomes maladaptive such as in chronic pain.

The scoping review method used to impartially identify relevant articles had its limitations. We used standard practices (a variety of MeSH terms and multiple databases) to capture the relevant literature and were able to define the major brain regions where relevant data exist. However, we found that many important studies were missed. We noted this often occurred when glycinergic signaling was studied as an extension of the main body of work. However, the majority of these papers were uncovered by consulting the reference lists of articles identified by the scoping review, as indicated in the PRISMA schema under the heading of "additional records included post-search" (Figure 3, 81% of total studies included in the review). Thus, although we cannot claim to have captured all studies investigating glycinergic signaling in pain-related brain regions, we are confident that we have identified the bulk of existing evidence. We hope that this review serves to stimulate interest and encourage researchers across multiple domains to consider possible roles for supraspinal glycine receptors and glycinergic signaling in their future studies.

AUTHOR CONTRIBUTIONS

Caitlin Fenech: Conceptualization; data curation; formal analysis; methodology; writing – original draft; writing – review and editing. **Bryony L. Winters:** Investigation; writing – original draft; writing – review and editing. **Yo Otsu:** Conceptualization; investigation; writing – original draft; writing – review and editing. **Karin R. Aubrey:** Conceptualization; funding acquisition; investigation; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

Karin Aubrey is currently a handling editor for the Journal of Neurochemistry.

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DATA AVAILABILITY STATEMENT

Data derived from public domain resources as indicated in the methods.

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REFERENCES

Abe, T., Shimoda, T., Urade, M., Hasegawa, M., Sugiyo, S., & Takemura, M. (2013). c-Fos induction in the brainstem following electrical stimulation of the trigeminal ganglion of chronically mandibular nerve-transected rats. *Somatosensory & Motor Research*, *30*(4), 175–184. https://doi.org/10.3109/08990220.2013.790805

Aggleton, J. P., O'Mara, S. M., Vann, S. D., Wright, N. F., Tsanov, M., & Erichsen, J. T. (2010). Hippocampal-anterior thalamic pathways for memory: Uncovering a network of direct and indirect actions. *The European Journal of Neuroscience*, *31*(12), 2292–2307. https://doi.org/10.1111/j.1460-9568.2010.07251.x

Al-Khater, K. M., & Todd, A. J. (2009). Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area. *The Journal of Comparative Neurology*, *515*(6), 629–646. https://doi.org/10.1002/cne.22081

Andermann, F., Keene, D. L., Andermann, E., & Quesney, L. F. (1980). Startle disease or hyperekplexia: Further delineation of the syndrome. *Brain*, *103*(4), 985–997. https://doi.org/10.1093/brain/103.4.985

Ankri, L., Husson, Z., Pietrajtis, K., Proville, R., Léna, C., Yarom, Y., Dieudonné, S., & Uusisaari, M. Y. (2015). A novel inhibitory nucleo-cortical circuit controls cerebellar Golgi cell activity. *eLife*, *4*, e06262. https://doi.org/10.7554/eLife.06262

Apkarian, A. V., Bushnell, M. C., Treede, R.-D., & Zubieta, J.-K. (2005). Human brain mechanisms of pain perception and regulation in health and disease. *European Journal of Pain*, *9*(4), 463. https://doi.org/10.1016/j.ejpain.2004.11.001

Apostolides, P. F., & Trussell, L. O. (2013). Rapid, activity-independent turnover of vesicular transmitter content at a mixed glycine/GABA synapse. *Journal of Neuroscience*, *33*(11), 4768–4781. https://doi.org/10.1523/JNEUROSCI.5555-12.2013

Araki, T., Yamano, M., Murakami, T., Wanaka, A., Betz, H., & Tohyama, M. (1988). Localization of glycine receptors in the rat central nervous system: An immunocytochemical analysis using monoclonal antibody. *Neuroscience*, *25*(2), 613–624. https://doi.org/10.1016/0306-4522(88)90263-1

Araya, A., Gallegos, S., Viveros, R., San Martin, L., Muñoz, B., Harvey, R. J., Zeilhofer, H. U., & Aguayo, L. G. (2021). Presence of ethanol-sensitive and ethanol-insensitive glycine receptors in the ventral tegmental area and prefrontal cortex in mice. *British Journal of Pharmacology*, *178*(23), 4691–4707. https://doi.org/10.1111/bph.15649

Assareh, N., Fenech, C., Power, R., Uddin, M. N., Otsu, Y., & Aubrey, K. R. (2023). Bidirectional modulation of nociception by GlyT2$^+$ neurons in the ventrolateral periaqueductal gray. *Eneuro*, *10*(6). https://doi.org/10.1523/eneuro.0069-23.2023

Aubrey, K. R., Rossi, F. M., Ruivo, R., Alboni, S., Bellenchi, G. C., Le Goff, A., Gasnier, B., & Supplisson, S. (2007). The transporters GlyT2 and VIAAT cooperate to determine the vesicular glycinergic phenotype. *The Journal of Neuroscience*, *27*(23), 6273–6281. https://doi.org/10.1523/jneurosci.1024-07.2007

Aubrey, K. R., & Supplisson, S. (2018). Heterogeneous signaling at GABA and glycine co-releasing terminals. *Frontiers in Synaptic Neuroscience*, *10*(40). https://doi.org/10.3389/fnsyn.2018.00040

Aubrey, K. R., Vandenberg, R. J., & Clements, J. D. (2005). Dynamics of forward and reverse transport by the glial glycine transporter, glyt1b. *Biophysical Journal*, *89*(3), 1657–1668. https://doi.org/10.1529/biophysj.105.061572

Avendaño, C., Machín, R., Bermejo, P. E., & Lagares, A. (2005). Neuron numbers in the sensory trigeminal nuclei of the rat: A GABA- and glycine-immunocytochemical and stereological analysis. *Journal of Comparative Neurology*, *493*(4), 538–553. https://doi.org/10.1002/cne.20778

Azhdari Zarmehri, H., Semnanian, S., Fathollahi, Y., Erami, E., Khakpay, R., Azizi, H., & Rohampour, K. (2011). Intra-periaqueductal gray matter microinjection of orexin-a decreases formalin-induced nociceptive behaviors in adult male rats. *The Journal of Pain*, *12*(2), 280–287. https://doi.org/10.1016/j.jpain.2010.09.006

Azhdari-Zarmehri, H., Semnanian, S., & Fathollahi, Y. (2014). Orexin-a microinjection into the rostral ventromedial medulla causes antinociception on formalin test. *Pharmacology Biochemistry and Behavior*, *122*, 286–290. https://doi.org/10.1016/j.pbb.2014.03.017

Bae, J. Y., Mah, W., Rah, J. C., Park, S. K., & Bae, Y. C. (2016). Expression of glycine receptor alpha 3 in the rat trigeminal neurons and central boutons in the brainstem. *Brain Structure and Function*, *221*(9), 4601–4613. https://doi.org/10.1007/s00429-016-1190-4

Baer, K., Waldvogel, H. J., Faull, R. L., & Rees, M. I. (2009). Localization of glycine receptors in the human forebrain, brainstem, and cervical spinal cord: An immunohistochemical review. *Frontiers in Molecular Neuroscience*, *2*, 25. https://doi.org/10.3389/neuro.02.025.2009

Bagley, E. E., & Ingram, S. L. (2020). Endogenous opioid peptides in the descending pain modulatory circuit. *Neuropharmacology*, *173*, 108131. https://doi.org/10.1016/j.neuropharm.2020.108131

Bagnall, M. W., Zingg, B., Sakatos, A., Moghadam, S. H., Zeilhofer, H. U., & Du Lac, S. (2009). Glycinergic projection neurons of the cerebellum. *Journal of Neuroscience*, *29*(32), 10104–10110. https://doi.org/10.1523/JNEUROSCI.2087-09.2009

Bandler, R., & Shipley, M. T. (1994). Columnar organization in the midbrain periaqueductal gray: Modules for emotional expression? *Trends in Neurosciences*, *17*(9), 379–389. https://doi.org/10.1016/0166-2236(94)90047-7

Bang, J. Y., Sunstrum, J. K., Garand, D., Parfitt, G. M., Woodin, M., Inoue, W., & Kim, J. (2022). Hippocampal-hypothalamic circuit controls context-dependent innate defensive responses. *eLife*, *11*, e74736. https://doi.org/10.7554/eLife.74736

Bannai, M., & Kawai, N. (2012). New therapeutic strategy for amino acid medicine: Glycine improves the quality of sleep. *Journal of Pharmacological Sciences*, *118*(2), 145–148. https://doi.org/10.1254/jphs.11R04FM

Barik, A., Thompson, J. H., Seltzer, M., Ghitani, N., & Chesler, A. T. (2018). A brainstem-spinal circuit controlling nocifensive behavior. *Neuron*, *100*(6), 1491–1503, e1493. https://doi.org/10.1016/j.neuron.2018.10.037

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell*, *139*(2), 267–284. https://doi.org/10.1016/j.cell.2009.09.028

Basbaum, A. I., & Fields, H. L. (1984). Endogenous pain control systems: Brainstem spinal pathways and endorphin circuitry. *Annual Review of Neuroscience*, *7*(1), 309–338. https://doi.org/10.1146/annurev.ne.07.030184.001521

Batten, T. F. C., Pow, D. V., & Saha, S. (2010). Co-localisation of markers for glycinergic and GABAergic neurones in rat nucleus of the solitary tract: Implications for co-transmission. *Journal of Chemical*

Neuroanatomy, *40*(2), 160–176. https://doi.org/10.1016/j.jchemneu.2010.04.001

Baumann, O., Borra, R. J., Bower, J. M., Cullen, K. E., Habas, C., Ivry, R. B., Leggio, M., Mattingley, J. B., Molinari, M., Moulton, E. A., Paulin, M. G., Pavlova, M. A., Schmahmann, J. D., & Sokolov, A. A. (2015). Consensus paper: The role of the cerebellum in perceptual processes. *The Cerebellum*, *14*(2), 197–220. https://doi.org/10.1007/s12311-014-0627-7

Beato, M., Groot-Kormelink, P. J., Colquhoun, D., & Sivilotti, L. G. (2004). The activation mechanism of alpha1 homomeric glycine receptors. *The Journal of Neuroscience*, *24*(4), 895–906. https://doi.org/10.1523/jneurosci.4420-03.2004

Beckers, A. B., van Oudenhove, L., Weerts, Z., Jacobs, H. I. L., Priovoulos, N., Poser, B. A., Ivanov, D., Gholamrezaei, A., Aziz, Q., Elsenbruch, S., Masclee, A. A. M., & Keszthelyi, D. (2022). Evidence for engagement of the nucleus of the solitary tract in processing intestinal chemonociceptive input irrespective of conscious pain response in healthy humans. *Pain*, *163*(8), 1520–1529. https://doi.org/10.1097/j.pain.0000000000002538

Behbehani, M., Park, M., & Clement, M. (1988). Interactions between the lateral hypothalamus and the periaqueductal gray. *The Journal of Neuroscience*, *8*(8), 2780–2787. https://doi.org/10.1523/jneurosci.08-08-02780.1988

Bliss, T. V. P., Collingridge, G. L., Kaang, B.-K., & Zhuo, M. (2016). Synaptic plasticity in the anterior cingulate cortex in acute and chronic pain. *Nature Reviews Neuroscience*, *17*(8), 485–496. https://doi.org/10.1038/nrn.2016.68

Blom, S. M., Pfister, J. P., Santello, M., Senn, W., & Nevian, T. (2014). Nerve injury-induced neuropathic pain causes disinhibition of the anterior cingulate cortex. *The Journal of Neuroscience*, *34*(17), 5754–5764. https://doi.org/10.1523/jneurosci.3667-13.2014

Borrus, D. S., Grover, C. J., Smith, G. D. C., & Del Negro, C. A. (2020). Role of synaptic inhibition in the coupling of the respiratory rhythms that underlie eupnea and sigh behaviors. *Eneuro*, *7*(3), 1–12. https://www.eneuro.org/content/7/3/ENEURO.0302-19.2020

Boscan, P., Pickering, A. E., & Paton, J. F. R. (2002). The nucleus of the solitary tract: An integrating station for nociceptive and cardiorespiratory afferents. *Experimental Physiology*, *87*(2), 259–266. https://doi.org/10.1113/eph8702353

Bossi, S., Dhanasobhon, D., Ellis-Davies, G. C. R., Frontera, J., de Brito Van Velze, M., Lourenco, J., Murillo, A., Lujan, R., Casado, M., Perez-Otano, I., Bacci, A., Popa, D., Paoletti, P., & Rebola, N. (2022). GluN3A excitatory glycine receptors control adult cortical and amygdalar circuits. *Neuron*, *110*(15), 2438–2454, e2438. https://doi.org/10.1016/j.neuron.2022.05.016

Bossi, S., Pizzamiglio, L., & Paoletti, P. (2023). Excitatory GluN1/GluN3A glycine receptors (eGlyRs) in brain signaling. *Trends in Neurosciences*, *46*(8), 667–681. https://doi.org/10.1016/j.tins.2023.05.002

Bouâouda, H., & Jha, P. K. (2023). Orexin and MCH neurons: Regulators of sleep and metabolism. *Frontiers in Neuroscience*, *17*, 1230428. https://doi.org/10.3389/fnins.2023.1230428

Buccitelli, C., & Selbach, M. (2020). mRNAs, proteins and the emerging principles of gene expression control. *Nature Reviews. Genetics*, *21*(10), 630–644. https://doi.org/10.1038/s41576-020-0258-4

Burstein, R., Cliffer, K., & Giesler, J. (1987). Direct somatosensory projections from the spinal cord to the hypothalamus and telencephalon. *The Journal of Neuroscience*, *7*(12), 4159–4164. https://doi.org/10.1523/jneurosci.07-12-04159.1987

Burstein, R., Cutrer, M. F., & Yarnitsky, D. (2000). The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. *Brain*, *123*(Pt 8), 1703–1709. https://doi.org/10.1093/brain/123.8.1703

Callister, R., & Graham, B. (2010). Early history of glycine receptor biology in mammalian spinal cord circuits. *Frontiers in Molecular Neuroscience*, *3*. https://doi.org/10.3389/fnmol.2010.00013

Capelli, P., Pivetta, C., Soledad Esposito, M., & Arber, S. (2017). Locomotor speed control circuits in the caudal brainstem. *Nature*, *551*(7680), 373–377. https://doi.org/10.1038/nature24064

Ceder, M. M., Weman, H. M., Johansson, E., Henriksson, K., Magnusson, K. A., Roman, E., & Lagerström, M. C. (2023). The glycine receptor alpha 3 subunit mRNA expression shows sex-dependent differences in the adult mouse brain. *BMC Neuroscience*, *24*(1), 32. https://doi.org/10.1186/s12868-023-00800-9

Cerritelli, S., Hirschberg, S., Hill, R., Balthasar, N., & Pickering, A. E. (2016). Activation of brainstem pro-opiomelanocortin neurons produces Opioidergic analgesia, bradycardia and bradypnoea. *PLoS One*, *11*(4), e0153187. https://doi.org/10.1371/journal.pone.0153187

Çetereisi, D., Kramvis, I., Gebuis, T., van der Loo, R. J., Gouwenberg, Y., Mansvelder, H. D., Li, K. W., Smit, A. B., & Spijker, S. (2019). Gpr158 deficiency impacts hippocampal CA1 neuronal excitability, dendritic architecture, and affects spatial learning. *Frontiers in Cellular Neuroscience*, *13*, 465. https://doi.org/10.3389/fncel.2019.00465

Chang, J., Song, Z., Wei, S., Zhou, Y., Ju, J., Yao, P., Jiang, Y., Jin, H., Chi, X., & Li, N. (2023). Expression mapping and functional analysis of orphan G-protein-coupled receptor GPR158 in the adult mouse brain using a GPR158 transgenic mouse. *Biomolecules*, *13*(3). https://doi.org/10.3390/biom13030479

Chattipakorn, S. C., & McMahon, L. L. (2002). Pharmacological characterization of glycine-gated chloride currents recorded in rat hippocampal slices. *Journal of Neurophysiology*, *87*(3), 1515–1525. https://doi.org/10.1152/jn.00365.2001

Chattipakorn, S. C., & McMahon, L. L. (2003). Strychnine-sensitive glycine receptors depress hyperexcitability in rat dentate gyrus. *Journal of Neurophysiology*, *89*(3), 1339–1342. https://doi.org/10.1152/jn.00908.2002

Chen, C. H., Newman, L. N., Stark, A. P., Bond, K. E., Zhang, D., Nardone, S., Vanderburg, C. R., Nadaf, N. M., Yao, Z., Mutume, K., Flaquer, I., Lowell, B. B., Macosko, E. Z., & Regehr, W. G. (2023). A Purkinje cell to parabrachial nucleus pathway enables broad cerebellar influence over the forebrain. *Nature Neuroscience*, *26*(11), 1929–1941. https://doi.org/10.1038/s41593-023-01462-w

Chen, Q., Roeder, Z., Li, M.-H., Zhang, Y., Ingram, S. L., & Heinricher, M. M. (2017). Optogenetic evidence for a direct circuit linking nociceptive transmission through the parabrachial complex with pain-modulating neurons of the rostral ventromedial medulla (RVM). *Eneuro*, *4*(3). https://doi.org/10.1523/eneuro.0202-17.2017

Chen, Y., Chen, A.-q., Luo, X.-q., Guo, L.-x., Tang, Y., Bao, C.-j., Lin, L., & Lin, C. (2014). Hippocampal NR2B-containing NMDA receptors enhance long-term potentiation in rats with chronic visceral pain. *Brain Research*, *1570*, 43–53. https://doi.org/10.1016/j.brainres.2014.05.001

Chéry, N., & De Koninck, Y. (1999). Junctional versus extrajunctional glycine and GABA(a) receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. *The Journal of Neuroscience*, *19*(17), 7342. https://doi.org/10.1523/JNEUROSCI.19-17-07342.1999

Chiang, M. C., Bowen, A., Schier, L. A., Tupone, D., Uddin, O., & Heinricher, M. M. (2019). Parabrachial complex: A hub for pain and aversion. *The Journal of Neuroscience*, *39*(42), 8225–8230. https://doi.org/10.1523/JNEUROSCI.1162-19.2019

Chiang, M. C., Nguyen, E. K., Canto-Bustos, M., Papale, A. E., Oswald, A. M., & Ross, S. E. (2020). Divergent neural pathways emanating from the lateral parabrachial nucleus mediate distinct components of the pain response. *Neuron*, *106*(6), 927–939, e925. https://doi.org/10.1016/j.neuron.2020.03.014

Cho, J. H., Deisseroth, K., & Bolshakov, V. Y. (2013). Synaptic encoding of fear extinction in mPFC-amygdala circuits. *Neuron*, *80*(6), 1491–1507. https://doi.org/10.1016/j.neuron.2013.09.025

Choi, K.-H., Nakamura, M., & Jang, I.-S. (2013). Presynaptic glycine receptors increase GABAergic neurotransmission in rat periaqueductal gray neurons. *Neural Plasticity*, *2013*, 954302. https://doi.org/10.1155/2013/954302

Ciabarra, A. M., Sullivan, J. M., Gahn, L. G., Pecht, G., Heinemann, S., & Sevarino, K. A. (1995). Cloning and characterization of chi-1: A developmentally regulated member of a novel class of the ionotropic glutamate receptor family. *The Journal of Neuroscience*, *15*(10), 6498–6508. https://doi.org/10.1523/jneurosci.15-10-06498.1995

Cinelli, E., Iovino, L., Bongianni, F., Pantaleo, T., & Mutolo, D. (2016). GABAA- and glycine-mediated inhibitory modulation of the cough reflex in the caudal nucleus tractus solitarii of the rabbit. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *311*(3), L570–L580. https://doi.org/10.1152/ajplung.00205.2016

Da Silva, J. T., & Seminowicz, D. A. (2019). Neuroimaging of pain in animal models: A review of recent literature. *Pain Reports*, *4*(4), e732. https://doi.org/10.1097/pr9.0000000000000732

Dafny, N., Dong, W. Q., Prieto-Gomez, C., Reyes-Vazquez, C., Stanford, J., & Qiao, J. T. (1996). Lateral hypothalamus: Site involved in pain modulation. *Neuroscience*, *70*(2), 449–460. https://doi.org/10.1016/0306-4522(95)00358-4

Danglot, L., Rostaing, P., Triller, A., & Bessis, A. (2004). Morphologically identified glycinergic synapses in the hippocampus. *Molecular and Cellular Neuroscience*, *27*(4), 394–403. https://doi.org/10.1016/j.mcn.2004.05.007

Danober, L., & Pape, H. C. (1998). Strychnine-sensitive glycine responses in neurons of the lateral amygdala: An electrophysiological and immunocytochemical characterization. *Neuroscience*, *85*(2), 427–441. https://doi.org/10.1016/S0306-4522(97)00648-9

de la Rocha-Muñoz, A., Núñez, E., Arribas-González, E., López-Corcuera, B., Aragón, C., & de Juan-Sanz, J. (2019). E3 ubiquitin ligases LNX1 and LNX2 are major regulators of the presynaptic glycine transporter GlyT2. *Scientific Reports*, *9*(1), 14944. https://doi.org/10.1038/s41598-019-51301-x

Del Negro, C. A., Funk, G. D., & Feldman, J. L. (2018). Breathing matters. *Nature Reviews Neuroscience*, *19*(6), 351–367. https://doi.org/10.1038/s41583-018-0003-6

Delaney, A. J., Esmaeili, A., Sedlak, P. L., Lynch, J. W., & Sah, P. (2010). Differential expression of glycine receptor subunits in the rat basolateral and central amygdala. *Neuroscience Letters*, *469*(2), 237–242. https://doi.org/10.1016/j.neulet.2009.12.003

Deng, J., Zhou, H., Lin, J. K., Shen, Z. X., Chen, W. Z., Wang, L. H., Li, Q., Mu, D., Wei, Y. C., Xu, X. H., & Sun, Y. G. (2020). The parabrachial nucleus directly channels spinal nociceptive signals to the intralaminar thalamic nuclei, but not the amygdala. *Neuron*, *107*(5), 909–923, e906. https://doi.org/10.1016/j.neuron.2020.06.017

Denk, F., McMahon, S. B., & Tracey, I. (2014). Pain vulnerability: A neurobiological perspective. *Nature Neuroscience*, *17*(2), 192–200. https://doi.org/10.1038/nn.3628

Dufour, A., Tell, F., Kessler, J., & Baude, A. (2010). Mixed GABA-glycine synapses delineate a specific topography in the nucleus tractus solitarii of adult rat. *Journal of Physiology*, *588*(7), 1097–1115. https://doi.org/10.1113/jphysiol.2009.184838

Dugué, G. P., Dumoulin, A., Triller, A., & Dieudonné, S. (2005). Target-dependent use of Coreleased inhibitory transmitters at central synapses. *The Journal of Neuroscience*, *25*(28), 6490. https://doi.org/10.1523/JNEUROSCI.1500-05.2005

Dumontier, D., Mailhes-Hamon, C., Supplisson, S., & Dieudonné, S. (2023). Neurotransmitter content heterogeneity within an interneuron class shapes inhibitory transmission at a central synapse. *Frontiers in Cellular Neuroscience*, *16*. https://doi.org/10.3389/fncel.2022.1060189

Dumoulin, A., Triller, A., & Dieudonné, S. (2001). IPSC kinetics at identified GABAergic and mixed GABAergic and Glycinergic synapses onto cerebellar Golgi cells. *The Journal of Neuroscience*, *21*(16), 6045. https://doi.org/10.1523/JNEUROSCI.21-16-06045.2001

Ezure, K., & Tanaka, I. (2004). GABA, in some cases together with glycine, is used as the inhibitory transmitter by pump cells in the Hering-Breuer reflex pathway of the rat. *Neuroscience*, *127*(2), 409–417. https://doi.org/10.1016/j.neuroscience.2004.05.032

Ezure, K., Tanaka, I., & Kondo, M. (2003). Glycine is used as a transmitter by decrementing expiratory neurons of the ventrolateral medulla in the rat. *Journal of Neuroscience*, *23*(26), 8941–8948. https://doi.org/10.1523/jneurosci.23-26-08941.2003

Ezure, K., Tanaka, I., & Saito, Y. (2003). Activity of brainstem respiratory neurones just before the expiration-inspiration transition in the rat. *The Journal of Physiology*, *547*(Pt 2), 629–640. https://doi.org/10.1113/jphysiol.2002.032805

Findlay, G. S., Phelan, R., Roberts, M. T., Homanics, G. E., Bergeson, S. E., Lopreato, G. F., Mihic, S. J., Blednov, Y. A., & Harris, R. A. (2003). Glycine receptor knock-in mice and hyperekplexia-like phenotypes: Comparisons with the null mutant. *Journal of Neuroscience*, *23*(22), 8051–8059. https://doi.org/10.1523/JNEUROSCI.23-22-08051.2003

Finnegan, T., Li, D.-P., Chen, S.-R., & Pan, H.-L. (2004). Activation of μ opioid receptors inhibits synaptic inputs to spinally projecting rostral ventromedial medulla neuro. *The Journal of Pharmacology and Experimental Therapeutics*, *309*(2), 476–483. https://doi.org/10.1124/jpet.103.064808

Fitzek, S., Baumgärtner, U., Fitzek, C., Magerl, W., Urban, P., Thömke, F., Marx, J., Treede, R.-D., Stoeter, P., & Hopf, H. C. (2001). Mechanisms and predictors of chronic facial pain in lateral medullary infarction. *Annals of Neurology*, *49*(4), 493–500. https://doi.org/10.1002/ana.99

Flor, K. C., Barnett, W. H., Karlen-Amarante, M., Molkov, Y. I., & Zoccal, D. B. (2020). Inhibitory control of active expiration by the Bötzinger complex in rats. *The Journal of Physiology*, *598*(21), 4969–4994. https://doi.org/10.1113/jp280243

Foster, E., Wildner, H., Tudeau, L., Haueter, S., Ralvenius, W. T., Jegen, M., Johannssen, H., Hosli, L., Haenraets, K., Ghanem, A., Conzelmann, K. K., Bosl, M., & Zeilhofer, H. U. (2015). Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch. *Neuron*, *85*(6), 1289–1304. https://doi.org/10.1016/j.neuron.2015.02.028

Frontera, J. L., Baba Aissa, H., Sala, R. W., Mailhes-Hamon, C., Georgescu, I. A., Léna, C., & Popa, D. (2020). Bidirectional control of fear memories by cerebellar neurons projecting to the ventrolateral periaqueductal grey. *Nature Communications*, *11*(1), 5207. https://doi.org/10.1038/s41467-020-18953-0

Ghirardini, E., Wadle, S. L., Augustin, V., Becker, J., Brill, S., Hammerich, J., Seifert, G., & Stephan, J. (2018). Expression of functional inhibitory neurotransmitter transporters GlyT1, GAT-1, and GAT-3 by astrocytes of inferior colliculus and hippocampus. *Molecular Brain*, *11*(1), 4. https://doi.org/10.1186/s13041-018-0346-y

Gomeza, J., Hulsmann, S., Ohno, K., Eulenburg, V., Szoke, K., Richter, D., & Betz, H. (2003). Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. *Neuron*, *40*(4), 785–796. http://ac.els-cdn.com/S089662730300672X/1-s2.0-S089662730300672X-main.pdf?_tid=61312956-39aa-11e5-9295-00000aab0f6c&acdnat=1438584144_4078c94da799a727876d4a092e2dc34e

Gomeza, J., Ohno, K., Hulsmann, S., Armsen, W., Eulenburg, V., Richter, D. W., Laube, B., & Betz, H. (2003). Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. *Neuron*, *40*(4), 797–806. http://ac.els-cdn.com/S0896627303006731/1-s2.0-S0896627303006731-main.pdf?_tid=b811a934-f8fb-11e4-b40b-00000aacb35e&acdnat=1431472253_fd38f3120bacac496d451c763130811d

Graham, B. A., Schofield, P. R., Sah, P., Margrie, T. W., & Callister, R. J. (2006). Distinct physiological mechanisms underlie altered glycinergic synaptic transmission in the murine mutants spastic, spasmodic, and oscillator. *Journal of Neuroscience*, *26*(18), 4880–4890. 10.1523%2FJNEUROSCI.3991-05.2006

Grand, T., Abi Gerges, S., David, M., Diana, M. A., & Paoletti, P. (2018). Unmasking GluN1/GluN3A excitatory glycine NMDA receptors. *Nature Communications*, *9*(1), 4769. https://doi.org/10.1038/s41467-018-07236-4

Groemer, T. W., Triller, A., Zeilhofer, H. U., Becker, K., Eulenburg, V., & Becker, C. M. (2022). Nociception in the glycine receptor deficient mutant mouse spastic. *Frontiers in Molecular Neuroscience*, *15*, 832490. https://doi.org/10.3389/fnmol.2022.832490

Grudzinska, J., Schemm, R., Haeger, S., Nicke, A., Schmalzing, G., Betz, H., & Laube, B. (2005). The β subunit determines the ligand binding properties of synaptic glycine receptors. *Neuron*, *45*(5), 727–739. https://doi.org/10.1016/j.neuron.2005.01.028

Gupta, R., Koscik, T. R., Bechara, A., & Tranel, D. (2011). The amygdala and decision-making. *Neuropsychologia*, *49*(4), 760–766. https://doi.org/10.1016/j.neuropsychologia.2010.09.029

Hammond, D. L., Presley, R., Gogas, K. R., & Basbaum, A. I. (1992). Morphine or U-50,488 suppresses fos protein-like immunoreactivity in the spinal cord and nucleus tractus solitarii evoked by a noxious visceral stimulus in the rat. *Journal of Comparative Neurology*, *315*(2), 244–253. https://doi.org/10.1002/cne.903150210

Hansen, K. B., Yi, F., Perszyk, R. E., Furukawa, H., Wollmuth, L. P., Gibb, A. J., & Traynelis, S. F. (2018). Structure, function, and allosteric modulation of NMDA receptors. *The Journal of General Physiology*, *150*(8), 1081–1105. https://doi.org/10.1085/jgp.201812032

Hansen, K. B., Yi, F., Perszyk, R. E., Menniti, F. S., & Traynelis, S. F. (2017). NMDA receptors in the central nervous system. In N. Burnashev & P. Szepetowski (Eds.), *NMDA receptors: Methods and protocols* (pp. 1–80). Springer. https://doi.org/10.1007/978-1-4939-7321-7_1

Harvey, R. J., & Yee, B. K. (2013). Glycine transporters as novel therapeutic targets in schizophrenia, alcohol dependence and pain. *Nature Reviews Drug Discovery*, *12*(11), 866–885. https://doi.org/10.1038/nrd3893

Henson, M. A., Roberts, A. C., Salimi, K., Vadlamudi, S., Hamer, R. M., Gilmore, J. H., Jarskog, L. F., & Philpot, B. D. (2008). Developmental regulation of the NMDA receptor subunits, NR3A and NR1, in human prefrontal cortex. *Cerebral Cortex*, *18*(11), 2560–2573. https://doi.org/10.1093/cercor/bhn017

Herbert, H., Guthmann, A., Zafra, F., & Ottersen, O. P. (2000). Glycine, glycine receptor subunit and glycine transporters in the rat parabrachial and Kolliker-fuse nuclei. *Anatomy and Embryology (Berlin)*, *201*(4), 259–272. https://doi.org/10.1007/s004290050316

Ho, Y.-C., Lee, H.-J., Tung, L.-W., Liao, Y.-Y., Fu, S.-Y., Teng, S.-F., Liao, H.-T., Mackie, K., & Chiou, L.-C. (2011). Activation of orexin 1 receptors in the periaqueductal gray of male rats leads to antinociception via retrograde endocannabinoid (2-arachidonoylglycerol)-induced disinhibition. *The Journal of Neuroscience*, *31*(41), 14600–14610. https://doi.org/10.1523/jneurosci.2671-11.2011

Holt, M. K. (2022). The ins and outs of the caudal nucleus of the solitary tract: An overview of cellular populations and anatomical connections. *Journal of Neuroendocrinology*, *34*(6), e13132. https://doi.org/10.1111/jne.13132

Hondo, M., Furutani, N., Yamasaki, M., Watanabe, M., & Sakurai, T. (2011). Orexin neurons receive glycinergic innervations. *PLoS One*, *6*(9), e25076. https://doi.org/10.1371/journal.pone.0025076

Hossaini, M., Goos, J. A. C., Kohli, S. K., & Holstege, J. C. (2012). Distribution of glycine/GABA neurons in the ventromedial medulla with descending spinal projections and evidence for an ascending glycine/GABA projection. *PLoS One*, *7*(4), e35293. https://doi.org/10.1371/journal.pone.0035293

Hsueh, B., Chen, R., Jo, Y., Tang, D., Raffiee, M., Kim, Y. S., Inoue, M., Randles, S., Ramakrishnan, C., Patel, S., Kim, D. K., Liu, T. X., Kim, S. H., Tan, L., Mortazavi, L., Cordero, A., Shi, J., Zhao, M., Ho, T. T., … Deisseroth, K. (2023). Cardiogenic control of affective behavioural state. *Nature*, *615*(7951), 292–299. https://doi.org/10.1038/s41586-023-05748-8

Husson, Z., Rousseau, C. V., Broll, I., Zeilhofer, H. U., & Dieudonné, S. (2014). Differential GABAergic and glycinergic inputs of inhibitory interneurons and Purkinje cells to principal cells of the cerebellar nuclei. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *34*(28), 9418–9431. https://doi.org/10.1523/JNEUROSCI.0401-14.2014

Jafari, H., Courtois, I., Van den Bergh, O., Vlaeyen, J. W. S., & Van Diest, I. (2017). Pain and respiration: a systematic review. *Pain*, *158*(6), 995–1006. https://doi.org/10.1097/j.pain.0000000000000865

Jennings, J. H., Ung, R. L., Resendez, S. L., Stamatakis, A. M., Taylor, J. G., Huang, J., Veleta, K., Kantak, P. A., Aita, M., Shilling-Scrivo, K., Ramakrishnan, C., Deisseroth, K., Otte, S., & Stuber, G. D. (2015). Visualizing hypothalamic network dynamics for appetitive and consummatory behaviors. *Cell*, *160*(3), 516–527. https://doi.org/10.1016/j.cell.2014.12.026

Jia, S., Rybalchenko, N., Kunwar, K., Farmer, G. E., Jr., Little, J. T., Toney, G. M., & Cunningham, J. T. (2022). Chronic intermittent hypoxia enhances glycinergic inhibition in nucleus tractus solitarius. *Journal of Neurophysiology*, *128*(6), 1383–1394. https://doi.org/10.1152/jn.00241.2022

Johnson, J. W., & Ascher, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, *325*(6104), 529–531. https://doi.org/10.1038/325529a0

Jonas, P., Bischofberger, J., & Sandkuhler, J. (1998). Corelease of two fast neurotransmitters at a central synapse. *Science*, *281*(5375), 419–424. http://www.ncbi.nlm.nih.gov/pubmed/9665886

Kajiyama, S., Kawamoto, M., Shiraishi, S., Gaus, S., Matsunaga, A., Suyama, H., & Yuge, O. (2005). Spinal orexin-1 receptors mediate anti-hyperalgesic effects of intrathecally-administered orexins in diabetic neuropathic pain model rats. *Brain Research*, *1044*(1), 76–86. https://doi.org/10.1016/j.brainres.2005.03.007

Karnani, M. M., Venner, A., Jensen, L. T., Fugger, L., & Burdakov, D. (2011). Direct and indirect control of orexin/hypocretin neurons by glycine receptors. *Journal of Physiology*, *589*(3), 639–651. https://doi.org/10.1113/jphysiol.2010.198457

Keay, K., & Bandler, R. (2008). Emotional and behavioral significance of the pain signal and the role of the midbrain periaqueductal gray (PAG). In R. H. Masland, T. D. Albright, T. D. Albright, R. H. Masland, P. Dallos, D. Oertel, S. Firestein, G. K. Beauchamp, M. C. Bushnell, A. I. Basbaum, J. H. Kaas, & E. P. Gardner (Eds.), *The senses: A comprehensive reference* (pp. 627–634). Academic Press. doi:10.1016/B978-012370880-9.00184-5

Keay, K. A., & Bandler, R. (2015). Chapter 10—periaqueductal gray. In G. Paxinos (Ed.), *The rat nervous system* (Fourth ed., pp. 207–221). Academic Press. https://doi.org/10.1016/B978-0-12-374245-2.00010-3

Keck, T., Mrsic-Flogel, T. D., Vaz Afonso, M., Eysel, U. T., Bonhoeffer, T., & Hübener, M. (2008). Massive restructuring of neuronal circuits during functional reorganization of adult visual cortex. *Nature Neuroscience*, *11*(10), 1162–1167. https://doi.org/10.1038/nn.2181

Keck, T., & White, J. A. (2009). Glycinergic inhibition in the hippocampus. *Reviews in the Neurosciences*, *20*(1), 13–22. https://doi.org/10.1515/REVNEURO.2009.20.1.13

Keller, A. F., Coull, J. A., Chery, N., Poisbeau, P., & De Koninck, Y. (2001). Region-specific developmental specialization of GABA-glycine cosynapses in laminas I-II of the rat spinal dorsal horn. *The Journal of Neuroscience*, *21*(20), 7871–7880.

Khrimian, L., Obri, A., Ramos-Brossier, M., Rousseaud, A., Moriceau, S., Nicot, A. S., Mera, P., Kosmidis, S., Karnavas, T., Saudou, F., Gao, X. B., Oury, F., Kandel, E., & Karsenty, G. (2017). Gpr158 mediates osteocalcin's regulation of cognition. *Journal of Experimental Medicine*, *214*(10), 2859–2873. https://doi.org/10.1084/jem.20171320

Kleckner, N. W., & Dingledine, R. (1988). Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science*, *241*(4867), 835–837.

Koutsikou, S., Crook, J. J., Earl, E. V., Leith, J. L., Watson, T. C., Lumb, B. M., & Apps, R. (2014). Neural substrates underlying fear-evoked freezing: The periaqueductal grey–cerebellar link. *The Journal of Physiology*, *592*(10), 2197–2213. https://doi.org/10.1113/jphysiol.2013.268714

Kubo, T., & Kihara, M. (1987). Evidence for the presence of GABAergic and glycine-like systems responsible for cardiovascular control in the nucleus tractus solitarii of the rat. *Neuroscience Letters*, *74*(3), 331–336. https://doi.org/10.1016/0304-3940(87)90319-3

Kuner, R., & Kuner, T. (2021). Cellular circuits in the brain and their modulation in acute and chronic pain. *Physiological Reviews*, *101*(1), 213–258. https://doi.org/10.1152/physrev.00040.2019

Laboute, T., Zucca, S., Holcomb, M., Patil, D. N., Garza, C., Wheatley, B. A., Roy, R. N., Forli, S., & Martemyanov, K. A. (2023). Orphan receptor GPR158 serves as a metabotropic glycine receptor: mGlyR. *Science*, *379*(6639), 1352–1358. https://doi.org/10.1126/science.add7150

Lau, B. K., & Vaughan, C. W. (2014). Descending modulation of pain: The GABA disinhibition hypothesis of analgesia. *Current Opinion in Neurobiology*, *29*, 159–164. https://doi.org/10.1016/j.conb.2014.07.010

Lau, B. K., Winters, B. L., & Vaughan, C. W. (2020). Opioid presynaptic disinhibition of the midbrain periaqueductal grey descending analgesic pathway. *British Journal of Pharmacology*, *177*(10), 2320–2332. https://doi.org/10.1111/bph.14982

Lawrenson, C., Bares, M., Kamondi, A., Kovács, A., Lumb, B., Apps, R., Filip, P., & Manto, M. (2018). The mystery of the cerebellum: Clues from experimental and clinical observations. *Cerebellum & Ataxias*, *5*(1), 8. https://doi.org/10.1186/s40673-018-0087-9

Lee, E. A., Cho, J. H., Choi, I. S., Nakamura, M., Park, H. M., Lee, J. J., Lee, M. G., Choi, B. J., & Jang, I. S. (2009). Presynaptic glycine receptors facilitate spontaneous glutamate release onto hilar neurons in the rat hippocampus. *Journal of Neurochemistry*, *109*(1), 275–286. https://doi.org/10.1111/j.1471-4159.2009.05960.x

Lee, S., Zhao, Y. Q., Ribeiro-da-Silva, A., & Zhang, J. (2010). Distinctive response of CNS glial cells in oro-facial pain associated with injury, infection and inflammation. *Molecular Pain*, *6*, 79. https://doi.org/10.1186/1744-8069-6-79

Legendre, P. (2001). The glycinergic inhibitory synapse. *Cellular and Molecular Life Sciences: CMLS*, *58*(5), 760–793. https://doi.org/10.1007/PL00000899

Leknes, S., & Tracey, I. (2008). A common neurobiology for pain and pleasure. *Nature Reviews Neuroscience*, *9*(4), 314–320. https://doi.org/10.1038/nrn2333

Lewis, J. W., Baldrighi, G., & Akil, H. (1987). A possible interface between autonomic function and pain control: Opioid analgesia and the nucleus tractus solitarius. *Brain Research*, *424*(1), 65–70. https://doi.org/10.1016/0006-8993(87)91193-0

Li, J.-N., & Sheets, P. L. (2020). Spared nerve injury differentially alters parabrachial monosynaptic excitatory inputs to molecularly specific neurons in distinct subregions of the central amygdala. *Pain*, *161*(1), 166–176. https://doi.org/10.1097/j.pain.0000000000001691

Li, S.-B., & de Lecea, L. (2020). The hypocretin (orexin) system: From a neural circuitry perspective. *Neuropharmacology*, *167*, 107993. https://doi.org/10.1016/j.neuropharm.2020.107993

Liang, S.-H., Yin, J.-B., Sun, Y., Bai, Y., Zhou, K.-X., Zhao, W.-J., Wang, W., Dong, Y.-L., & Li, Y.-Q. (2016). Collateral projections from the lateral parabrachial nucleus to the paraventricular thalamic nucleus and the central amygdaloid nucleus in the rat. *Neuroscience Letters*, *629*, 245–250. https://doi.org/10.1016/j.neulet.2016.07.017

Lieske, S. P., Thoby-Brisson, M., Telgkamp, P., & Ramirez, J. M. (2000). Reconfiguration of the neural network controlling multiple breathing patterns: Eupnea, sighs and gasps. *Nature Neuroscience*, *3*(6), 600–607. https://doi.org/10.1038/75776

Liu, S., Ye, M., Pao, G. M., Song, S. M., Jhang, J., Jiang, H., Kim, J.-H., Kang, S. J., Kim, D.-I., & Han, S. (2022). Divergent brainstem opioidergic pathways that coordinate breathing with pain and emotions. *Neuron*, *110*(5), 857–873, e859. https://doi.org/10.1016/j.neuron.2021.11.029

Liu, Y., Huang, D., Wen, R., Chen, X., & Yi, H. (2015). Glycine receptor-mediated inhibition of medial prefrontal cortical pyramidal cells. *Biochemical and Biophysical Research Communications*, *456*(2), 666–669. https://doi.org/10.1016/j.bbrc.2014.12.014

Lynch, J. W. (2004). Molecular structure and function of the glycine receptor chloride channel. *Physiological Reviews*, *84*(4), 1051–1095. https://doi.org/10.1152/physrev.00042.2003

Lynch, J. W. (2009). Native glycine receptor subtypes and their physiological roles. *Neuropharmacology*, *56*(1), 303–309. https://doi.org/10.1016/j.neuropharm.2008.07.034

Maione, S., Marabese, I., Rossi, F., Berrino, L., Palazzo, E., & Trabace, L. (2000). Effects of persistent nociception on periaqueductal gray glycine release. *Neuroscience*, *97*(2), 311–316. https://doi.org/10.1016/s0306-4522(00)00076-2

Malmierca, E., Martin, Y. B., & Nuñez, A. (2012). Inhibitory control of nociceptive responses of trigeminal spinal nucleus cells by somatosensory corticofugal projection in rat. *Neuroscience*, *221*, 115–124. https://doi.org/10.1016/j.neuroscience.2012.07.003

Malosio, M. L., Marquèze-Pouey, B., Kuhse, J., & Betz, H. (1991). Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *The EMBO Journal*, *10*(9), 2401–2409. https://doi.org/10.1002/j.1460-2075.1991.tb07779.x

Marchenko, V., Koizumi, H., Mosher, B., Koshiya, N., Tariq, M. F., Bezdudnaya, T. G., Zhang, R., Molkov, Y. I., Rybak, I. A., & Smith, J. C. (2016). Perturbations of respiratory rhythm and pattern by disrupting synaptic inhibition within pre-Bötzinger and Bötzinger complexes. *Eneuro*, *3*(2). https://doi.org/10.1523/eneuro.0011-16.2016

Martina, M., Turcotte, M. E. B., Halman, S., Tsai, G., Tiberi, M., Coyle, J. T., & Bergeron, R. (2005). Reduced glycine transporter type 1 expression leads to major changes in glutamatergic neurotransmission of CA1 hippocampal neurones in mice. *Journal of Physiology*, *563*(3), 777–793. https://doi.org/10.1113/jphysiol.2004.080655

Mason, P. (2005). Ventromedial medulla: Pain modulation and beyond. *Journal of Comparative Neurology*, *493*(1), 2–8. https://doi.org/10.1002/cne.20751

Matheus, M. G., Nogueira, R. L., Carobrez, A. P., Graeff, F. G., & Guimarães, F. S. (1994). Anxiolytic effect of glycine antagonists microinjected into the dorsal periaqueductal grey. *Psychopharmacology*, *113*(3–4), 565–569. https://doi.org/10.1007/BF02245241

Mathiasen, M. L., O'Mara, S. M., & Aggleton, J. P. (2020). The anterior thalamic nuclei and nucleus reuniens: So similar but so different. *Neuroscience & Biobehavioral Reviews*, *119*, 268–280. https://doi.org/10.1016/j.neubiorev.2020.10.006

McCracken, L. M., Lowes, D. C., Salling, M. C., Carreau-Vollmer, C., Odean, N. N., Blednov, Y. A., Betz, H., Harris, R. A., & Harrison, N. L. (2017). Glycine receptor α3 and α2 subunits mediate tonic and exogenous agonist-induced currents in forebrain. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(34), E7179–e7186. https://doi.org/10.1073/pnas.1703839114

McGarry, L. M., & Carter, A. G. (2017). Prefrontal cortex drives distinct projection neurons in the basolateral amygdala. *Cell Reports*, *21*(6), 1426–1433. https://doi.org/10.1016/j.celrep.2017.10.046

McIntire, S. L., Reimer, R. J., Schuske, K., Edwards, R. H., & Jorgensen, E. M. (1997). Identification and characterization of the vesicular GABA transporter. *Nature*, *389*(6653), 870–876. https://doi.org/10.1038/39908

Melin, C., Jacquot, F., Vitello, N., Dallel, R., & Artola, A. (2017). Different processing of meningeal and cutaneous pain information in the spinal trigeminal nucleus caudalis. *Cephalalgia*, *37*(12), 1189–1201. https://doi.org/10.1177/0333102416673204

Mercer Lindsay, N., Chen, C., Gilam, G., Mackey, S., & Scherrer, G. (2021). Brain circuits for pain and its treatment. *Science Translational Medicine*, *13*(619), eabj7360. https://doi.org/10.1126/scitranslmed.abj7360

Miranda, C. O., Hegedüs, K., Wildner, H., Zeilhofer, H. U., & Antal, M. (2022). Morphological and neurochemical characterization of glycinergic neurons in laminae I–IV of the mouse spinal dorsal horn. *Journal of Comparative Neurology*, *530*(3), 607–626. https://doi.org/10.1002/cne.25232

Moraga-Cid, G., San Martín, V. P., Lara, C. O., Muñoz, B., Marileo, A. M., Sazo, A., Muñoz-Montesino, C., Fuentealba, J., Castro, P. A., Guzmán, L., Burgos, C. F., Zeilhofer, H. U., Aguayo, L. G., Corringer, P.-J., & Yévenes, G. E. (2020). Modulation of glycine receptor single-channel conductance by intracellular phosphorylation. *Scientific Reports*, *10*(1), 4804. https://doi.org/10.1038/s41598-020-61677-w

Morales, F. R., Sampogna, S., Rampon, C., Luppi, P. H., & Chase, M. H. (2006). Brainstem glycinergic neurons and their activation during active (rapid eye movement) sleep in the cat. *Neuroscience*, *142*(1), 37–47. https://doi.org/10.1016/j.neuroscience.2006.05.066

Morgado-Valle, C., Baca, S. M., & Feldman, J. L. (2010). Glycinergic Pacemaker Neurons in PreBötzinger Complex of Neonatal Mouse. *The Journal of Neuroscience*, *30*(10), 3634–3639. https://doi.org/10.1523/jneurosci.3040-09.2010

Mori, M., Gahwiler, B. H., & Gerber, U. (2002). Beta-alanine and taurine as endogenous agonists at glycine receptors in rat hippocampus in vitro. *The Journal of Physiology*, *539*(Pt 1), 191–200. https://doi.org/10.1113/jphysiol.2001.013147

Morton, D. L., Sandhu, J. S., & Jones, A. K. (2016). Brain imaging of pain: State of the art. *Journal of Pain Research*, *9*, 613–624. https://doi.org/10.2147/jpr.S60433

Moulton, E. A., Schmahmann, J. D., Becerra, L., & Borsook, D. (2010). The cerebellum and pain: Passive integrator or active participator? *Brain Research Reviews*, *65*(1), 14–27. https://doi.org/10.1016/j.brainresrev.2010.05.005

Mueller, H. T., & Meador-Woodruff, J. H. (2005). Distribution of the NMDA receptor NR3A subunit in the adult pig-tail macaque brain. *Journal of Chemical Neuroanatomy*, *29*(3), 157–172. https://doi.org/10.1016/j.jchemneu.2004.11.002

Mukhtarov, M., Ragozzino, D., & Bregestovski, P. (2005). Dual Ca^{2+} modulation of glycinergic synaptic currents in rodent hypoglossal motoneurones. *Journal of Physiology*, *569*(3), 817–831. https://doi.org/10.1113/jphysiol.2005.094862

Muller, E., Bakkar, W., Martina, M., Sokolovski, A., Wong, A. Y., Legendre, P., & Bergeron, R. (2013). Vesicular storage of glycine in glutamatergic terminals in mouse hippocampus. *Neuroscience*, *242*, 110–127. https://doi.org/10.1016/j.neuroscience.2013.03.007

Murillo, A., Navarro, A. I., Puelles, E., Zhang, Y., Petros, T. J., & Pérez-Otaño, I. (2020). Temporal dynamics and neuronal specificity of Grin3a expression in the mouse forebrain. *Cerebral Cortex*, *31*(4), 1914–1926. https://doi.org/10.1093/cercor/bhaa330

Mutso, A. A., Radzicki, D., Baliki, M. N., Huang, L., Banisadr, G., Centeno, M. V., Radulovic, J., Martina, M., Miller, R. J., & Apkarian, A. V. (2012). Abnormalities in hippocampal functioning with persistent pain. *The Journal of Neuroscience*, *32*(17), 5747–5756. https://doi.org/10.1523/jneurosci.0587-12.2012

Neumann, N., Domin, M., Schmidt, C.-O., & Lotze, M. (2023). Chronic pain is associated with less grey matter volume in the anterior cingulum, anterior and posterior insula and hippocampus across three different chronic pain conditions. *European Journal of Pain*, *27*(10), 1239–1248. https://doi.org/10.1002/ejp.2153

Obolenskaya, M., Dotsenko, V., Martsenyuk, O., Ralchenko, S., Krupko, O., Pastukhov, A., Filimonova, N., Starosila, D., Chernykh, S., & Borisova, T. (2021). A new insight into mechanisms of interferon alpha neurotoxicity: Expression of GRIN3A subunit of NMDA receptors and NMDA-evoked exocytosis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *110*, 110317. https://doi.org/10.1016/j.pnpbp.2021.110317

Oliva, C. A., Stehberg, J., Barra, R., & Mariqueo, T. (2022). Neuropathic pain induces interleukin-1β sensitive bimodal Glycinergic activity in the central amygdala. *International Journal of Molecular Sciences*, *23*, 7356. https://doi.org/10.3390/ijms23137356

Ong, W. Y., Stohler, C. S., & Herr, D. R. (2019). Role of the prefrontal cortex in pain processing. *Molecular Neurobiology*, *56*(2), 1137–1166. https://doi.org/10.1007/s12035-018-1130-9

Orlandi, C., Posokhova, E., Masuho, I., Ray, T. A., Hasan, N., Gregg, R. G., & Martemyanov, K. A. (2012). GPR158/179 regulate G protein signaling by controlling localization and activity of the RGS7 complexes. *The Journal of Cell Biology*, *197*(6), 711–719. https://doi.org/10.1083/jcb.201202123

Orlandi, C., Xie, K., Masuho, I., Fajardo-Serrano, A., Lujan, R., & Martemyanov, K. A. (2015). Orphan receptor GPR158 is an allosteric modulator of RGS7 catalytic activity with an essential role in dictating its expression and localization in the brain. *The Journal of Biological Chemistry*, *290*(22), 13622–13639. https://doi.org/10.1074/jbc.M115.645374

Otsu, Y., & Aubrey, K. R. (2022). Kappa opioids inhibit the GABA/glycine terminals of rostral ventromedial medulla projections in the superficial dorsal horn of the spinal cord. *The Journal of Physiology*, *600*(18), 4187–4205. https://doi.org/10.1113/JP283021

Otsu, Y., Darcq, E., Pietrajtis, K., Mátyás, F., Schwartz, E., Bessaih, T., Abi Gerges, S., Rousseau, C. V., Grand, T., Dieudonné, S., Paoletti, P., Acsády, L., Agulhon, C., Kieffer, B. L., & Diana, M. A. (2019). Control of aversion by glycine-gated GluN1/GluN3A NMDA receptors in the adult medial habenula. *Science*, *366*(6462), 250–254. https://doi.org/10.1126/science.aax1522

Palazzo, E., Guida, F., Migliozzi, A., Gatta, L., Marabese, I., Luongo, L., Rossi, C., Novellis, V. D., Fernández-Sánchez, E., Soukupova, M., Zafra, F., & Maione, S. (2009). Intraperiaqueductal gray glycine and d-serine exert dual effects on rostral ventromedial medulla on- and off-cell activity and Thermoceptive threshold in the rat. *Journal of Neurophysiology*, *102*(6), 3169–3179. https://doi.org/10.1152/jn.00124.2009

Palmiter, R. D. (2018). The parabrachial nucleus: CGRP neurons function as a general alarm. *Trends in Neurosciences*, *41*(5), 280–293. https://doi.org/10.1016/j.tins.2018.03.007

Patel, N. M., Jozsa, F., & Das, J. M. (2024). Neuroanatomy, spinal trigeminal nucleus. In *StatPearls*. StatPearls Publishing LLC.

Patra, P. H., Tench, B., Hitrec, T., Holmes, F., Drake, R., Cerritelli, S., Spanswick, D., & Pickering, A. E. (2023). Pro-opiomelanocortin neurons in the nucleus of the solitary tract mediate endorphinergic endogenous analgesia in mice. *Pain*, *164*(5), 1051–1066. https://doi.org/10.1097/j.pain.0000000000002802

Pauli, J. L., Chen, J. Y., Basiri, M. L., Park, S., Carter, M. E., Sanz, E., McKnight, G. S., Stuber, G. D., & Palmiter, R. D. (2022). Molecular and anatomical characterization of parabrachial neurons and their axonal projections. *eLife*, *11*, e81868. https://doi.org/10.7554/eLife.81868

Pellicer, F., López-Avila, A., Coffeen, U., Manuel Ortega-Legaspi, J., & Angel, R. D. (2007). Taurine in the anterior cingulate cortex diminishes neuropathic nociception: A possible interaction with the glycine(a) receptor. *European Journal of Pain*, *11*(4), 444–451. https://doi.org/10.1016/j.ejpain.2006.06.003

Petrovich, G. D. (2018). Lateral hypothalamus as a motivation-cognition interface in the control of feeding behavior. *Frontiers in Systems Neuroscience*, *12*, 14. https://doi.org/10.3389/fnsys.2018.00014

Peyron, R., Schneider, F., Faillenot, I., Convers, P., Barral, F. G., Garcia-Larrea, L., & Laurent, B. (2004). An fMRI study of cortical representation of mechanical allodynia in patients with neuropathic pain. *Neurology*, *63*(10), 1838–1846. https://doi.org/10.1212/01.wnl.0000144177.61125.85

Phua, S. C., Tan, Y. L., Kok, A. M. Y., Senol, E., Chiam, C. J. H., Lee, C.-Y., Peng, Y., Lim, A. T. J., Mohammad, H., Lim, J.-X., & Fu, Y. (2021). A distinct parabrachial–to–lateral hypothalamus circuit for motivational suppression of feeding by nociception. *Science Advances*, *7*(19), eabe4323. https://doi.org/10.1126/sciadv.abe4323

Pi, G., Gao, D., Wu, D., Wang, Y., Lei, H., Zeng, W., Gao, Y., Yu, H., Xiong, R., Jiang, T., Li, S., Wang, X., Guo, J., Zhang, S., Yin, T., He, T., Ke, D., Li, R., Li, H., … Wang, J. Z. (2020). Posterior basolateral amygdala to ventral hippocampal CA1 drives approach behaviour to exert an

anxiolytic effect. *Nature Communications*, *11*(1), 183. https://doi.org/10.1038/s41467-019-13919-3

Pinto, M., Lima, D., & Tavares, I. (2007). Neuronal activation at the spinal cord and medullary pain control centers after joint stimulation: A c-fos study in acute and chronic articular inflammation. *Neuroscience*, *147*(4), 1076–1089. https://doi.org/10.1016/j.neuroscience.2007.05.019

Poyatos, I., Ponce, J., Aragon, C., Gimenez, C., & Zafra, F. (1997). The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons. *Molecular Brain Research*, *49*(1–2), 63–70. https://doi.org/10.1016/s0169-328x(97)00124-1

Price, D. D. (2000). Psychological and neural mechanisms of the affective dimension of pain. *Science*, *288*(5472), 1769–1772. https://doi.org/10.1126/science.288.5472.1769

Probst, A., Cortés, R., & Palacios, J. M. (1986). The distribution of glycine receptors in the human brain. A light microscopic autoradiographic study using [3H]strychnine. *Neuroscience*, *17*(1), 11–35. https://doi.org/10.1016/0306-4522(86)90222-8

Radhakrishnan, R., & Sluka, K. A. (2009). Increased glutamate and decreased glycine release in the rostral ventromedial medulla during induction of a pre-clinical model of chronic widespread muscle pain. *Neuroscience Letters*, *457*(3), 141–145. https://doi.org/10.1016/j.neulet.2009.03.086

Rampon, C., Luppi, P. H., Fort, P., Peyron, C., & Jouvet, M. (1996). Distribution of glycine-immunoreactive cell bodies and fibers in the rat brain. *Neuroscience*, *75*(3), 737–755. https://doi.org/10.1016/0306-4522(96)00278-3

Rampon, C., Peyron, C., Gervasoni, D., Pow, D. V., Luppi, P.-H., & Fort, P. (1999). Origins of the glycinergic inputs to the rat locus coeruleus and dorsal raphe nuclei: A study combining retrograde tracing with glycine immunohistochemistry. *European Journal of Neuroscience*, *11*(3), 1058–1066. https://doi.org/10.1046/j.1460-9568.1999.00511.x

Raver, C., Uddin, O., Ji, Y., Li, Y., Cramer, N., Jenne, C., Morales, M., Masri, R., & Keller, A. (2020). An amygdalo-parabrachial pathway regulates pain perception and chronic pain. *The Journal of Neuroscience*, *40*(17), 3424–3442. https://doi.org/10.1523/jneurosci.0075-20.2020

Ressot, C., Collado, V., Molat, J. L., & Dallel, R. (2001). Strychnine alters response properties of trigeminal nociceptive neurons in the rat. *Journal of Neurophysiology*, *86*(6), 3069–3072. https://doi.org/10.1152/jn.2001.86.6.3069

Rinaman, L. (2010). Ascending projections from the caudal visceral nucleus of the solitary tract to brain regions involved in food intake and energy expenditure. *Brain Research*, *1350*, 18–34. https://doi.org/10.1016/j.brainres.2010.03.059

Roeder, Z., Chen, Q., Davis, S., Carlson, J. D., Tupone, D., & Heinricher, M. M. (2016). Parabrachial complex links pain transmission to descending pain modulation. *Pain*, *157*(12), 2697–2708. https://doi.org/10.1097/j.pain.0000000000000688

Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., & Kuncl, R. W. (1994). Localization of neuronal and glial glutamate transporters. *Neuron*, *13*(3), 713–725.

Rouch, I., Edjolo, A., Laurent, B., Pongan, E., Dartigues, J.-F., & Amieva, H. (2021). Association between chronic pain and long-term cognitive decline in a population-based cohort of elderly participants. *Pain*, *162*(2), 552–560. https://doi.org/10.1097/j.pain.0000000000002047

Rousseau, F., Aubrey, K. R., & Supplisson, S. (2008). The glycine transporter GlyT2 controls the dynamics of synaptic vesicle refilling in inhibitory spinal cord neurons. *The Journal of Neuroscience*, *28*(39), 9755–9768. https://doi.org/10.1523/jneurosci.0509-08.2008

Rudolph, S., Badura, A., Lutzu, S., Pathak, S. S., Thieme, A., Verpeut, J. L., Wagner, M. J., Yang, Y.-M., & Fioravante, D. (2023). Cognitive-affective functions of the cerebellum. *The Journal of Neuroscience*, *43*(45), 7554–7564. https://doi.org/10.1523/jneurosci.1451-23.2023

Saab, C. Y., & Willis, W. D. (2002). Cerebellar stimulation modulates the intensity of a visceral nociceptive reflex in the rat. *Experimental Brain Research*, *146*(1), 117–121. https://doi.org/10.1007/s00221-002-1107-8

Saab, C. Y., & Willis, W. D. (2003). The cerebellum: Organization, functions and its role in nociception. *Brain Research Reviews*, *42*(1), 85–95. https://doi.org/10.1016/S0165-0173(03)00151-6

Salling, M. C., & Harrison, N. L. (2014). Strychnine-sensitive glycine receptors on pyramidal neurons in layers II/III of the mouse prefrontal cortex are tonically activated. *Journal of Neurophysiology*, *112*(5), 1169–1178. https://doi.org/10.1152/jn.00714.2013

Samineni, V. K., Grajales-Reyes, J. G., Copits, B. A., O'Brien, D. E., Trigg, S. L., Gomez, A. M., Bruchas, M. R., & Gereau, R. W. (2017). Divergent modulation of nociception by glutamatergic and GABAergic neuronal subpopulations in the periaqueductal gray. *Eneuro*, *4*(2). https://doi.org/10.1523/ENEURO.0129-16.2017

Samineni, V. K., Grajales-Reyes, J. G., Sundaram, S. S., Yoo, J. J., & Gereau, R. W. (2019). Cell type-specific modulation of sensory and affective components of itch in the periaqueductal gray. *Nature Communications*, *10*(1), 4356. https://doi.org/10.1038/s41467-019-12316-0

Sato, K., Zhang, J. H., Saika, T., Sato, M., Tada, K., & Tohyama, M. (1991). Localization of glycine receptor alpha 1 subunit mRNA-containing neurons in the rat brain: An analysis using in situ hybridization histochemistry. *Neuroscience*, *43*(2–3), 381–395. https://doi.org/10.1016/0306-4522(91)90302-5

Schmahmann, J. D. (1996). From movement to thought: Anatomic substrates of the cerebellar contribution to cognitive processing. *Human Brain Mapping*, *4*(3), 174–198. https://doi.org/10.1002/(SICI)1097-0193(1996)4:3<174::AID-HBM3>3.0.CO;2-0

Schmitt, M. L., Coelho, W., Lopes-de-Souza, A. S., Guimarães, F. S., & Carobrez, A. P. (1995). Anxiogenic-like effect of glycine and D-serine microinjected into dorsal periaqueductal gray matter of rats. *Neuroscience Letters*, *189*(2), 93–96. https://doi.org/10.1016/0304-3940(95)11459-a

Schreihofer, A. M., Stornetta, R. L., & Guyenet, P. G. (1999). Evidence for glycinergic respiratory neurons: Botzinger neurons express mRNA for glycinergic transporter 2. *Journal of Comparative Neurology*, *407*(4), 583–597. https://doi.org/10.1002/(SICI)1096-9861(19990517)407:4<583::AID-CNE8>3.0.CO;2-E

Sellmeijer, J., Mathis, V., Hugel, S., Li, X. H., Song, Q., Chen, Q. Y., Barthas, F., Lutz, P. E., Karatas, M., Luthi, A., Veinante, P., Aertsen, A., Barrot, M., Zhuo, M., & Yalcin, I. (2018). Hyperactivity of anterior cingulate cortex areas 24a/24b drives chronic pain-induced anxiodepressive-like consequences. *The Journal of Neuroscience*, *38*(12), 3102–3115. https://doi.org/10.1523/jneurosci.3195-17.2018

Sessle, B. J. (2006). Mechanisms of oral somatosensory and motor functions and their clinical correlates. *Journal of Oral Rehabilitation*, *33*(4), 243–261. https://doi.org/10.1111/j.1365-2842.2006.01623.x

Shao, S., Zheng, Y., Fu, Z., Wang, J., Zhang, Y., Wang, C., Qi, X., Gong, T., Ma, L., Lin, X., Yu, H., Yuan, S., Wan, Y., Zhang, H., & Yi, M. (2023). Ventral hippocampal CA1 modulates pain behaviors in mice with peripheral inflammation. *Cell Reports*, *42*(1). https://doi.org/10.1016/j.celrep.2023.112017

Sheng, J., Liu, S., Wang, Y., Cui, R., & Zhang, X. (2017). The link between depression and chronic pain: Neural mechanisms in the brain. *Neural Plasticity*, *2017*, 9724371. https://doi.org/10.1155/2017/9724371

Sherman, D., Worrell, J. W., Cui, Y., & Feldman, J. L. (2015). Optogenetic perturbation of preBötzinger complex inhibitory neurons modulates respiratory pattern. *Nature Neuroscience*, *18*(3), 408–414. https://doi.org/10.1038/nn.3938

Simat, M., Parpan, F., & Fritsch, J. M. (2007). Heterogeneity of glycinergic and gabaergic interneurons in the granule cell layer of mouse cerebellum. *Journal of Comparative Neurology*, *500*(1), 71–83. https://doi.org/10.1002/cne.21142

Smith, J. C., Abdala, A. P., Koizumi, H., Rybak, I. A., & Paton, J. F. (2007). Spatial and functional architecture of the mammalian brain stem respiratory network: A hierarchy of three oscillatory mechanisms. *Journal of Neurophysiology*, *98*(6), 3370–3387. https://doi.org/10.1152/jn.00985.2007

Song, W., Chattipakorn, S. C., & McMahon, L. L. (2006). Glycine-gated chloride channels depress synaptic transmission in rat hippocampus. *Journal of Neurophysiology*, *95*(4), 2366–2379. https://doi.org/10.1152/jn.00386.2005

Stegemann, A., Liu, S., Retana Romero, O. A., Oswald, M. J., Han, Y., Beretta, C. A., Gan, Z., Tan, L. L., Wisden, W., Gräff, J., & Kuner, R. (2023). Prefrontal engrams of long-term fear memory perpetuate pain perception. *Nature Neuroscience*, *26*(5), 820–829. https://doi.org/10.1038/s41593-023-01291-x

Stroebel, D., Mony, L., & Paoletti, P. (2021). Glycine agonism in ionotropic glutamate receptors. *Neuropharmacology*, *193*, 108631. https://doi.org/10.1016/j.neuropharm.2021.108631

Stuber, G. D., & Wise, R. A. (2016). Lateral hypothalamic circuits for feeding and reward. *Nature Neuroscience*, *19*(2), 198–205. https://doi.org/10.1038/nn.4220

Sully, R., Mark, E., McNeely, W., Libenson, M., & Yang, J. (2001). Case records of the Massachusetts General Hospital. weekly clinicopathological exercises. Case 12-2001. A 16-year-old boy with an altered mental status and muscle rigidity. *The New England Journal of Medicine*, *344*(16), 1232–1239. https://doi.org/10.1056/nejm200104193441608

Sun, L., Liu, R., Guo, F., Wen, M.-q., Ma, X.-l., Li, K.-y., Sun, H., Xu, C.-l., Li, Y.-y., Wu, M.-y., Zhu, Z.-g., Li, X.-j., Yu, Y.-q., Chen, Z., Li, X.-y., & Duan, S. (2020). Parabrachial nucleus circuit governs neuropathic pain-like behavior. *Nature Communications*, *11*(1), 5974. https://doi.org/10.1038/s41467-020-19767-w

Supplisson, S., & Roux, M. J. (2002). Why glycine transporters have different stoichiometries. *FEBS Letters*, *529*(1), 93–101. https://doi.org/10.1016/S0014-5793(02)03251-9

Sutton, L. P., Orlandi, C., Song, C., Oh, W. C., Muntean, B. S., Xie, K., Filippini, A., Xie, X., Satterfield, R., Yaeger, J. D. W., Renner, K. J., Young, S. M., Jr., Xu, B., Kwon, H., & Martemyanov, K. A. (2018). Orphan receptor GPR158 controls stress-induced depression. *eLife*, *7*, e33273. https://doi.org/10.7554/eLife.33273

Tajerian, M., Hung, V., Nguyen, H., Lee, G., Joubert, L.-M., Malkovskiy, A. V., Zou, B., Xie, S., Huang, T.-T., & Clark, J. D. (2018). The hippocampal extracellular matrix regulates pain and memory after injury. *Molecular Psychiatry*, *23*(12), 2302–2313. https://doi.org/10.1038/s41380-018-0209-z

Talman, W. T., & Robertson, S. C. (1989). Glycine, like glutamate, microinjected into the nucleus tractus solitarii of rat decreases arterial pressure and heart rate. *Brain Research*, *477*(1), 7–13. https://doi.org/10.1016/0006-8993(89)91388-7

Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J.-I., Obata, K., & Kaneko, T. (2003). Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *Journal of Comparative Neurology*, *467*(1), 60–79. https://doi.org/10.1002/cne.10905

Tanaka, I., & Ezure, K. (2004). Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. *Neuroscience Research*, *49*(2), 165–178. https://doi.org/10.1016/j.neures.2004.02.007

Thek, K. R., Ong, S. J. M., Carter, D. C., Bassi, J. K., Allen, A. M., & McDougall, S. J. (2019). Extensive inhibitory gating of Viscerosensory signals by a sparse network of somatostatin neurons. *The Journal of Neuroscience*, *39*(41), 8038–8050. https://doi.org/10.1523/JNEUROSCI.3036-18.2019

Tijssen, M. A. J., Schoemaker, H. C., Edelbroek, P. J., Roos, R. A. C., Cohen, A. F., & van Dijk, J. G. (1997). The effects of clonazepam and vigabatrin in hyperekplexia. *Journal of the Neurological Sciences*, *149*(1), 63–67. https://doi.org/10.1016/S0022-510X(97)05378-1

Tonsfeldt, K. J., Suchland, K. L., Beeson, K. A., Lowe, J. D., Li, M. H., & Ingram, S. L. (2016). Sex differences in GABAA signaling in the periaqueductal gray induced by persistent inflammation. *The Journal of Neuroscience*, *36*(5), 1669–1681. https://doi.org/10.1523/jneurosci.1928-15.2016

Torres-Rodriguez, J. M., Wilson, T. D., Singh, S., Torruella-Suárez, M. L., Chaudhry, S., Adke, A. P., Becker, J. J., Neugebauer, B., Lin, J. L., Martinez Gonzalez, S., Soler-Cedeño, O., & Carrasquillo, Y. (2024). The parabrachial to central amygdala pathway is critical to injury-induced pain sensitization in mice. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *49*, 508–520. https://doi.org/10.1038/s41386-023-01673-6

Tovote, P., Esposito, M. S., Botta, P., Chaudun, F., Fadok, J. P., Markovic, M., Wolff, S. B. E., Ramakrishnan, C., Fenno, L., Deisseroth, K., Herry, C., Arber, S., & Lüthi, A. (2016). Midbrain circuits for defensive behaviour. *Nature*, *534*(7606), 206–212. https://doi.org/10.1038/nature17996

Tsai, H.-W., & Davenport, P. W. (2014). Tracheal occlusion-evoked respiratory load compensation and inhibitory neurotransmitter expression in rats. *Journal of Applied Physiology*, *116*(8), 1006–1016. https://doi.org/10.1152/japplphysiol.01256.2013

Uchida, S., Soya, S., Saito, Y. C., Hirano, A., Koga, K., Tsuda, M., Abe, M., Sakimura, K., & Sakurai, T. (2021). A discrete Glycinergic neuronal population in the ventromedial medulla that induces muscle Atonia during REM sleep and cataplexy in mice. *The Journal of Neuroscience*, *41*(7), 1582. https://doi.org/10.1523/JNEUROSCI.0688-20.2020

Urien, L., Xiao, Z., Dale, J., Bauer, E. P., Chen, Z., & Wang, J. (2018). Rate and temporal coding mechanisms in the anterior cingulate cortex for pain anticipation. *Scientific Reports*, *8*(1), 8298. https://doi.org/10.1038/s41598-018-26518-x

Uusisaari, M., & Knöpfel, T. (2010). GlyT2+ neurons in the lateral cerebellar nucleus. *The Cerebellum*, *9*(1), 42–55. https://doi.org/10.1007/s12311-009-0137-1

Vaaga, C. E., Borisovska, M., & Westbrook, G. L. (2014). Dual-transmitter neurons: Functional implications of co-release and co-transmission. *Current Opinion in Neurobiology*, *29*, 25–32. https://doi.org/10.1016/j.conb.2014.04.010

Vaaga, C. E., Brown, S. T., & Raman, I. M. (2020). Cerebellar modulation of synaptic input to freezing-related neurons in the periaqueductal gray. *eLife*, *9*, e54302. https://doi.org/10.7554/eLife.54302

Valentinova, K., Acuña, M. A., Ntamati, N. R., Nevian, N. E., & Nevian, T. (2023). An amygdala-to-cingulate cortex circuit for conflicting choices in chronic pain. *Cell Reports*, *42*(10), 113125. https://doi.org/10.1016/j.celrep.2023.113125

van den Pol, A. N., & Gorcs, T. (1988). Glycine and glycine receptor immunoreactivity in brain and spinal cord. *Journal of Neuroscience*, *8*(2), 472–492. https://doi.org/10.1523/jneurosci.08-02-00472.1988

Vandenberg, R. J., Ryan, R. M., Carland, J. E., Imlach, W. L., & Christie, M. J. (2014). Glycine transport inhibitors for the treatment of pain. *Trends in Pharmacological Sciences*, *35*(8), 423–430. https://doi.org/10.1016/j.tips.2014.05.006

Varga, E., Farkas, E., Zséli, G., Kádár, A., Venczel, A., Kővári, D., Németh, D., Máté, Z., Erdélyi, F., Horváth, A., Szenci, O., Watanabe, M., Lechan, R. M., Gereben, B., & Fekete, C. (2019). Thyrotropin-releasing-hormone-synthesizing neurons of the hypothalamic paraventricular nucleus are inhibited by Glycinergic inputs. *Thyroid*, *29*(12), 1858–1868. https://doi.org/10.1089/thy.2019.0357

Vaughn, E., Eichhorn, S., Jung, W., Zhuang, X., & Dulac, C. (2022). Three-dimensional interrogation of cell types and instinctive behavior in

the periaqueductal gray. *bioRxiv* https://doi.org/10.1101/2022.06.27.497769

Veinante, P., Yalcin, I., & Barrot, M. (2013). The amygdala between sensation and affect: A role in pain. *Journal of Molecular Psychiatry*, *1*(1), 9. https://doi.org/10.1186/2049-9256-1-9

Vuilleumier, P. H., Besson, M., Desmeules, J., Arendt-Nielsen, L., & Curatolo, M. (2013). Evaluation of anti-Hyperalgesic and analgesic effects of two benzodiazepines in human experimental pain: A randomized placebo-controlled study. *PLoS One*, *8*(3), e43896. https://doi.org/10.1371/journal.pone.0043896

Vuilleumier, P. H., Fritsche, R., Schliessbach, J., Schmitt, B., Arendt-Nielsen, L., Zeilhofer, H. U., & Curatolo, M. (2018). Mutations affecting glycinergic neurotransmission in hyperekplexia increase pain sensitivity. *Brain*, *141*(1), 63–71. https://doi.org/10.1093/brain/awx289

Wang, C., Fujita, T., & Kumamoto, E. (2018a). Modulation by orexin a of spontaneous excitatory and inhibitory transmission in adult rat spinal substantia gelatinosa neurons. *Biochemical and Biophysical Research Communications*, *501*(1), 100–105. https://doi.org/10.1016/j.bbrc.2018.04.182

Wang, C., Fujita, T., & Kumamoto, E. (2018b). Orexin B modulates spontaneous excitatory and inhibitory transmission in lamina II neurons of adult rat spinal cord. *Neuroscience*, *383*, 114–128. https://doi.org/10.1016/j.neuroscience.2018.04.048

Wang, D., Wu, J.-H., Dong, Y.-X., & Li, Y.-Q. (2001). Synaptic connections between trigemino-parabrachial projection neurons and γ-aminobutyric acid- and glycine-immunoreactive terminals in the rat. *Brain Research*, *921*(1), 133–137. https://doi.org/10.1016/S0006-8993(01)03109-2

Wang, L.-H., Ding, W.-Q., & Sun, Y.-G. (2022). Spinal ascending pathways for somatosensory information processing. *Trends in Neurosciences*, *45*(8), 594–607. https://doi.org/10.1016/j.tins.2022.05.005

Weera, M. M., Shackett, R. S., Kramer, H. M., Middleton, J. W., & Gilpin, N. W. (2021). Central amygdala projections to lateral hypothalamus mediate avoidance behavior in rats. *The Journal of Neuroscience*, *41*(1), 61–72. https://doi.org/10.1523/jneurosci.0236-20.2020

Wilcox, S. L., Gustin, S. M., Macey, P. M., Peck, C. C., Murray, G. M., & Henderson, L. A. (2015). Anatomical changes at the level of the primary synapse in neuropathic pain: Evidence from the spinal trigeminal nucleus. *The Journal of Neuroscience*, *35*(6), 2508–2515. https://doi.org/10.1523/jneurosci.3756-14.2015

Winters, B. L., Lau, B. K., & Vaughan, C. W. (2022). Cannabinoids and opioids differentially target extrinsic and intrinsic GABAergic inputs onto the periaqueductal grey descending pathway. *The Journal of Neuroscience*, *42*(41), 7744–7756. https://doi.org/10.1523/jneurosci.0997-22.2022

Wong, H.-K., Liu, X.-B., Matos, M. F., Chan, S. F., Pérez-Otaño, I., Boysen, M., Cui, J., Nakanishi, N., Trimmer, J. S., Jones, E. G., Lipton, S. A., & Sucher, N. J. (2002). Temporal and regional expression of NMDA receptor subunit NR3A in the mammalian brain. *Journal of Comparative Neurology*, *450*(4), 303–317. https://doi.org/10.1002/cne.10314

Xu, T. L., & Gong, N. (2010). Glycine and glycine receptor signaling in hippocampal neurons: Diversity, function and regulation. *Progress in Neurobiology*, *91*(4), 349–361. https://doi.org/10.1016/j.pneurobio.2010.04.008

Yaksh, T. L. (1989). Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: Effects of modulatory receptor systems and excitatory amino acid antagonists. *Pain*, *37*(1), 111–123. https://doi.org/10.1016/0304-3959(89)90160-7

Yamadera, W., Inagawa, K., Chiba, S., Bannai, M., Takahashi, M., & Nakayama, K. (2007). Glycine ingestion improves subjective sleep quality in human volunteers, correlating with polysomnographic changes. *Sleep and Biological Rhythms*, *5*(2), 126–131. https://doi.org/10.1111/j.1479-8425.2007.00262.x

Yamamoto, T., Saito, O., Shono, K., Aoe, T., & Chiba, T. (2003). Antimechanical allodynic effect of intrathecal and intracerebroventricular injection of orexin-a in the rat neuropathic pain model. *Neuroscience Letters*, *347*(3), 183–186. https://doi.org/10.1016/S0304-3940(03)00716-X

Yamarick, W., Walson, P., & DiTraglia, J. (1992). Strychnine poisoning in an adolescent. *Journal of Toxicology. Clinical Toxicology*, *30*(1), 141–148. https://doi.org/10.3109/15563659208994455

Yamashita, T., & Yamanaka, A. (2017). Lateral hypothalamic circuits for sleep-wake control. *Current Opinion in Neurobiology*, *44*, 94–100. https://doi.org/10.1016/j.conb.2017.03.020

Yang, C. F., & Feldman, J. L. (2018). Efferent projections of excitatory and inhibitory preBötzinger complex neurons. *Journal of Comparative Neurology*, *526*(8), 1389–1402. https://doi.org/10.1002/cne.24415

Yasui, Y., Masaki, E., & Kato, F. (2011). Esmolol modulates inhibitory neurotransmission in the substantia gelatinosa of the spinal trigeminal nucleus of the rat. *BMC Anesthesiology*, *11*, 15. https://doi.org/10.1186/1471-2253-11-15

Zafra, F., Ibáñez, I., & Giménez, C. (2016). Glycinergic transmission: Glycine transporter GlyT2 in neuronal pathologies. *Neuronal Signaling*, *1*(1), NS20160009. https://doi.org/10.1042/NS20160009

Zeilhofer, H. U., Acuña, M. A., Gingras, J., & Yévenes, G. E. (2018). Glycine receptors and glycine transporters: Targets for novel analgesics? *Cellular and Molecular Life Sciences*, *75*(3), 447–465. https://doi.org/10.1007/s00018-017-2622-x

Zeilhofer, H. U., Studler, B., Arabadzisz, D., Schweizer, C., Ahmadi, S., Layh, B., Bösl, M. R., & Fritschy, J.-M. (2005). Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. *Journal of Comparative Neurology*, *482*(2), 123–141. https://doi.org/10.1002/cne.20349

Zhang, H. X., & Thio, L. L. (2007). Zinc enhances the inhibitory effects of strychnine-sensitive glycine receptors in mouse hippocampal neurons. *Journal of Neurophysiology*, *98*(6), 3666–3676. https://doi.org/10.1152/jn.00500.2007

Zhang, L.-H., Gong, N., Fei, D., Xu, L., & Xu, T.-L. (2008). Glycine uptake regulates hippocampal network activity via glycine receptor-mediated tonic inhibition. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *33*(3), 701–711. https://doi.org/10.1038/sj.npp.1301449

Zhang, Z.-Y., Guo, Z., Li, H.-L., He, Y.-T., Duan, X.-L., Suo, Z.-W., Yang, X., & Hu, X.-D. (2019). Ubiquitination and inhibition of glycine receptor by HUWE1 in spinal cord dorsal horn. *Neuropharmacology*, *148*, 358–365. https://doi.org/10.1016/j.neuropharm.2019.02.002

Zhu, H., & Gouaux, E. (2021). Architecture and assembly mechanism of native glycine receptors. *Nature*, *599*(7885), 513–517. https://doi.org/10.1038/s41586-021-04022-z

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Title: Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature

Authors: Caitlin Fenech[2,1], Bryony L. Winters[3,1] Yo Otsu[1,2] & Karin R. Aubrey[1,2*]

Supplementary Data 1. *Complete list of searching methodology.*

OVID MEDLINE(R) ALL:

1	exp Glycine/	38894
2	glycine*.mp.	89776
3	1 or 2	93128
4	exp Synaptic Transmission/	66988
5	neurotransmi*.mp.	124710
6	4 or 5	176552
7	3 and 6	6075
8	brain/ or brain stem/ or gray matter/ or limbic system/ or prosencephalon/ or white matter/	589787
9	7 and 8	823
10	exp Neurons/	578469
11	9 and 10	259

EMBASE:

1	exp glycine/	61102
2	glycine*.mp.	116462
3	1 or 2	116462
4	exp neurotransmission/	241292
5	neurotransmi*.mp.	173164
6	4 or 5	332039
7	3 and 6	8979
8	exp brain/	1598672
9	7 and 8	4050
10	exp nerve cell/	873760
11	9 and 10	1971

Supplementary Table 1: *List of papers identified by scoping search considering brain regions with no clear role in pain sensation or behaviors.*

Region	Suggested function	Experimental technique	Gly-Interrogation	Outcome	Reference
Nucleus ambiguus and dorsal vagal motor nucleus					
Cardiac vagal neurons in the nucleus ambiguus	Cardiac function	Electrophysiology	GlyR	Activation of the β1 subtype of the adrenergic receptor decreased glycinergic neurotransmission	Bateman et al. (2012)
Cardiac vagal neurons in the nucleus ambiguus	Cardiac function	Electrophysiology	GlyR	α1 adrenergic receptor activation increases glycinergic neurotransmission	Boychuk et al. (2011)
Cardiac vagal neurons in the nucleus ambiguus	Cardiac function	Electrophysiology	GlyR	Dopamine via D2-like (D2, D3, D4) receptors suppresses glycinergic input	Dyavanapalli et al. (2013)
Cardiac vagal neurons in the nucleus ambiguus	Cardiac function	Electrophysiology	GlyR	Dexmedetomdine, an α2 adrenergic receptor agonist, decreases inhibitory glycinergic neurotransmission	Sharp et al. (2014)
Cardiac vagal neurons in the nucleus ambiguus	Cardiac function	Optogenetics	GlyR	Optogenetic activation of adrenergic (α1 and β1) neurons in the locus coeruleus increased glycinergic neurotransmission to cardiac vagal neurons in mice	Wang et al. (2014)
Cardioinhibitory parasympathetic neurons in the nucleus ambiguus	Respiration	Immunoreactivity and electrophysiology	GlyR	These neurons are inhibited (by both GABA and glycine) during inspiration, however unlike GABA, glycine activity is not mediated by nicotinic receptors	Neff et al. (2003)
Dorsal vagal motor nucleus	Cardiac function	Microinjection of glycine	Glycine	Microinjection caused increase in arterial pressure and heart rate in rats, which is not modulated through cholinergic mechanisms	Talman et al. (1992)
Medial nucleus of the trapezoid body and other regions of the central auditory system					
Medial nucleus of the trapezoid body	Auditory system	Electrophysiology	GlyR	There is increased glycinergic activity in congentially deaf mice compared to naïve	Leao et al. (2004)
Medial nucleus of the trapezoid body	Auditory system	Electrophysiology	GlyR	Inhibitory inputs are mainly fast and phasic glycinergic synapses (in a Mongolian gerbil)	Mayer et al. (2014)
Medial nucleus of the trapezoid body	Auditory system	Electrophysiology	GlyR	In deaf oterferlin KO mice, projections to the lateral superior olive do not undergo synaptic maturation and therefore, these synapses develop under the guidance of acoustic experience	Müller et al. (2022)
Medial nucleus of the trapezoid body	Auditory system	Electrophysiology	GlyR	Medial olivocochlear neurons are inhibited (by both GABA and glycine) by neurons of the medial nucleus of the trapezoid body in mice	Torres Cadenas et al. (2020)
Medial nucleus of the trapezoid body	Auditory system	Immunoreactivity	GlyT2	In macaque monkeys, there is expression of GlyT2 in the medial nucleus of the trapezoid body and along with GAD67, in the superior olivary complex and ventral nucleus of lateral lemniscus	Ito et al. (2015)

Medial nucleus of trapezoid body	Autism spectrum disorder	Electrophysiology	GlyR	Neuroligin-4 (monogenetic cause of autism) KO mice have impaired glycinergic synaptic transmission in the MNTB	Zhang et al. (2018)
Dorsal cochlear nucleus	Auditory system	Electrophysiology	GlyR	Glycinergic neurotransmission is related to the onset of salicylate induced tinnitus and this is mediated by the opening of ATP-sensitive potassium channels in the dorsal cochlear nucleus	de Siqueira et al. (2022)
Superior olivary complex	Auditory system	Electrophysiology and immunoreactivity	GlyR, glycine	Both GABAergic and glycinergic inhibition mediates interaural time disparities in chickens	Coleman et al. (2011)
Lateral superior olive	Auditory system	Electrophysiology	GlyR	Stimulation of the projection to the gerbil lateral superior olive shows a switch from GABAergic to glycinergic transmission during postnatal development	Kotak et al. (1998)
Lateral superior olive	Auditory system	Electrophysiology	GlyR	In mice shortly after hearing onset, glycinergic projections to the lateral superior olive displayed short term depression and synaptic attenuation in a frequency dependent matter	Kramer et al. (2014)
Calyx of Held	Auditory system	Electrophysiology	GlyR	At postnatal day 25 in rats, glutamatergic signals can be suppressed when preceded by glycinergic transmission	Awatramani et al. (2004)
Dorsal nucleus of the lateral lemniscus	Auditory system	Electrophysiology	GlyR	These neurons (used for sound localisation) in chickens have functional glycine receptors	Curry and Lu (2016)
Nucleus magnocellularis and nucleus laminaris	Auditory system	Electrophysiology	GlyR	Functional glycine receptors are expressed in these areas of the avian brainstem and glycine is released due to high frequency stimulation	Fischl and Burger (2014)
Central auditory system (many regions)	Auditory system	immunoreactivity	GlyT2	GlyT2 immunoreactivity found in all regions of the rat auditory system except the auditory cortex	Friauf et al. (1999)
Hypoglossal motoneurons and other regions relating to eye/head movement					
Hypoglossal motoneurons	Orofacial movement	Electrophysiology	GlyR	GABA and glycine are co-released from presynaptic terminals to hypoglossal motoneurons in rats	O'Brien and Berger (1999)
Hypoglossal motoneurons	Orofacial movement	Electrophysiology	GlyR	GABAergic and glycinergic input to hypoglossal motoneurons, in rats, is inhibited by presynaptic GABA(B) receptors	O'Brien et al. (2004)
Hypoglossal motoneurons	Orofacial movement	Electrophysiology	GlyR	Following perinatal nicotine exposure, glycinergic neurotransmission to hypoglossal motoneurons is enhanced only after acetylcholine receptor activation in rats	Wollman et al. (2018)
Facial and hypoglossal motoneurons	Orofacial movement	Immunoreactivity	Glycine	Retrograde tracer in facial and hypoglossal nucleus showed shared distribution of GABA-like and glycine-like input to these regions in rats	Li et al. (1998)
Supratrigeminal region	Orofacial movement	Electrophysiology	GlyR	In neonatal rats, glycine postsynaptic responses (as well as GABA and glutamate) activate jaw opening and closing motoneurons, but inhibit these neurons in juvenile rats	Nakamura et al. (2008)
Trigeminal motoneurons	Orofacial movement	Electrophysiology	GlyR	GABA and glycine are co-transmitted at the jaw opening and closing motoneurons, but this changed throughout development in rats	Noguchi et al. (2022)

Prepositus hypoglossi nucleus	Gaze holding	Electrophysiology	GlyT2	Most inhibitory neurons in this region use either GABA or both GABA and glycine as neurotransmitters in rats	Shino et al. (2011)
Hypoglossal motoneurons	Respiration	Electrophysiology	VIAAT, GlyT2	Conditional VIAAT KO in GlyT2::Cre animals, are not viable at birth as well as an absence of glycinergic and GABAergic signalling in hypoglossal motoneurons	Rahman et al. (2015)
BötC and hypoglossal nerve	Tongue muscle movement	Electrophysiology	Glycine	In rats with *in utero* nicotine exposure, microinjection of glycine into the hypoglossal nerve further reduced burst amplitude and altered nerve burst frequency in the PreBötC	Jaiswal et al. (2016)
Hypoglossal nucleus and dorsal vagal motor nucleus	Rett syndrome	Optogenetics	GlyR	Optogenetic activation of GAD expressing neurons in MeCP2 mice (Rett syndrome mutation) produce GABAergic and glycinergic inhibition of these regions (rely more on glycinergic)	Xing et al. (2021)
Hypoglossal motoneurons	Ethanol consumption	Electrophysiology	GlyR	Bath application of ethanol to hypoglossal motoneurons causes increased glycinergic activity in both neonates and juvenile rats	Sebe et al. (2003)
Superior salivatory nucleus	Orofacial movement	Electrophysiology	GlyR	Superior salivatory nucleus neurons that innervate the salivary glands and the tongue receive glycinergic inputs in rats	Mitoh et al. (2004)
Inferior olive	Coordinated movement	Electrophysiology, immunoreactivity and optogenetics	GlyR, GlyT2, vGAT	Vestibular nuclei to inferior olive projections are mediated by combined GABAergic and glycinergic synchronous transmission	Turecek and Regehr (2020)
Abducens motoneurons	Extraocular eye movement	Electrophysiology	GlyR	Inhibition of abducens motoneuron firing from the medial vestibular nucleus is achieved through corelease of GABA and glycine in rats	Russier et al. (2002)
Abducens neurons	Extraocular eye movement	Immunoreactivity	GlyT2, GlyRα1	Primate abducens neurons receive both glycinergic and GABAergic inputs	Mayadali et al. (2021)
Medial vestibular nucleus	Eye and head movement	Electrophysiology	GlyR	Type B medial vestibular neurons receive GABA(A)ergic and glycinergic inputs in isolation and combination	Camp et al. (2006)
Omnidirectional pause neurons in the raphe interpositus nucleus	Eye movement	Simulation modelling	GlyR	Glycinergic omnidirectional pause neurons inhibit the premotor burst neurons that are involved with saccadic eye movements	Optican (2008)
Other					
Ganglion cells in retina	Autism spectrum disorder	Electrophysiology and immunoreactivity	GlyR	Neuroligin-4 (monogenetic cause of autism) KO mice have impaired glycinergic synaptic transmission in ganglion cells in the retina and protein expression in the brainstem and spinal cord aligns with GlyR labelling	Hoon et al. (2011)
Phrenic nerves	Respiration	Electrophysiology	GlyRα1	Strychnine blockage of phrenic nerves in mutant oscillator mice (GlyRα1 mutation) does not cause respiratory failure	Busselberg et al. (2001)
Small ventral lateral neurons	Sleep	Electrophysiology and gene silencing (RNAi)	dGlyT (*Drosophila* glycine	RNAi-mediated downregulation of glycine transporter in these neurons increased circadian cycle length in flies	Frenkel et al. (2017)

			transporter)		
Suprachiasmatic nucleus	Sleep	Immunoreactivity and imaging	Glycine	Exogenous glycine administration in rats alleviated acute sleep disturbance via peripheral vasodilation	Kawai et al. (2015)
Pedunculopontine nucleus	Sleep	Electrophysiology	GlyR	Fast inhibitory synaptic transmission to these thalamic projecting neurons is modulated by both GABA(A) and glycine receptors in rats	Ye et al. (2010)
Ventromedial medulla	Sleep	Behaviour	vGAT	Rats with impaired inhibitory neurotransmission in this region resulted in REM sleep without atonia associated with abnormal and violent motor activity	Valencia Garcia et al. (2018)
Ventral medulla	Sleep	Optogenetics and chemogenetics	GlyR	Pathway from ventral medulla inhibit (GABA/glycine neurons) brainstem hypoglossal motoneurons and chemogenetic activation of ventral medulla GABA/glycine neurons produced inhibitory effect on tongue electromyographic activity in mice	Dergachev a et al. (2020)
Trigeminal motor nucleus	Sleep	Electrophysiology	Glycine	Blockade of the cystic fibrosis transmembrane regulator (partially responsible for REM sleep atonia) reduces glycinergic postsynaptic inhibitory actions in rats	Morales et al. (2011)
Basal forebrain	Neurodegenerative diseases	Immunoreactivity and electrophysiology	GlyT2, GlyR	Identified glycinergic inputs to cholinergic neurons in the basal forebrain from the raphe magnus and reticular formation of the pons in mice	Bardoczi et al. (2017)
Ventral tegmental area (VTA)	Ethanol consumption	Microinjection of glycine	Glycine	Causes a reduced intake of ethanol in rats chronically exposed to ethanol	Li et al. (2012)
Prefrontal Cortex (PFC), striatum, hippocampus and the nucleus accumbens	Ethanol consumption	Electrophysiology	GlyRα3	Tonically activated GlyRs (α3 subunit) are located in the PFC, striatum, hippocampus and the nucleus accumbens of mice	McCracken et al. (2017)
Unipolar brush cells (UBCs) in vestibulocerebellar lobes	Ethanol consumption	Electrophysiology	GlyR	Recreational concentrations of ethanol in mice increase glycinergic inhibition of UBCs	Richardson and Rossi (2017)
Systemic (not region specific)	Hypnosis induced by ethanol	Subcutaneous injection of strychnine	Strychnine	Reduced the number of rats that exhibited loss of righting reflex induced by ethanol	Ye et al. (2009)
Systemic	Ethanol consumption	Electrophysiology	GlyRα1	GMO mouse model with silent mutations affecting GlyRα1 displayed normal glycinergic transmission and reduction of loss of righting reflex induced by intoxicating dose of ethanol	Aguayo et al. (2014)

Supplementary Table 1 References:

Aguayo, L. G., Castro, P., Mariqueo, T., Muñoz, B., Xiong, W., Zhang, L., Lovinger, D. M., & Homanics, G. E. (2014). Altered Sedative Effects of Ethanol in Mice with α1 Glycine Receptor Subunits that are Insensitive to Gβγ Modulation. *Neuropsychopharmacology*, *39*(11), 2538-2548. https://doi.org/10.1038/npp.2014.100

Awatramani, G. B., Turecek, R., & Trussell, L. O. (2004). Inhibitory control at a synaptic relay [Research Support, U.S. Gov't, P.H.S.]. *Journal of Neuroscience*, *24*(11), 2643-2647. https://doi.org/10.1523/jneurosci.5144-03.2004

Bardoczi, Z., Pal, B., Koszeghy, A., Wilheim, T., Watanabe, M., Zaborszky, L., Liposits, Z., & Kallo, I. (2017). Glycinergic Input to the Mouse Basal Forebrain Cholinergic Neurons [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *Journal of Neuroscience*, *37*(39), 9534-9549. https://doi.org/https://dx.doi.org/10.1523/JNEUROSCI.3348-16.2017

Bateman, R. J., Boychuk, C. R., Philbin, K. E., & Mendelowitz, D. (2012). β adrenergic receptor modulation of neurotransmission to cardiac vagal neurons in the nucleus ambiguus [Article]. *Neuroscience*, *210*, 58-66. https://doi.org/10.1016/j.neuroscience.2012.02.033

Boychuk, C. R., Bateman, R. J., Philbin, K. E., & Mendelowitz, D. (2011). α1-adrenergic receptors facilitate inhibitory neurotransmission to cardiac vagal neurons in the nucleus ambiguus [Article]. *Neuroscience*, *193*, 154-161. https://doi.org/10.1016/j.neuroscience.2011.07.024

Busselberg, D., Bischoff, A. M., Becker, K., Becker, C. M., & Richter, D. W. (2001). The respiratory rhythm in mutant oscillator mice. *Neuroscience Letters*, *316(2)*, 99-102. https://doi.org/10.1016/s0304-3940(01)02382-5

Camp, A. J., Callister, R. J., & Brichta, A. M. (2006). Inhibitory synaptic transmission differs in mouse type A and B medial vestibular nucleus neurons in vitro. *Journal of Neurophysiology*, *95(5)*, 3208-3218. https://doi.org/https://dx.doi.org/10.1152/jn.01001.2005

Coleman, W. L., Fischl, M. J., Weimann, S. R., & Burger, R. M. (2011). GABAergic and glycinergic inhibition modulate monaural auditory response properties in the avian superior olivary nucleus. *Journal of Neurophysiology*, *105(5)*, 2405-2420. https://doi-org.ezproxy.library.sydney.edu.au/10.1152/jn.01088.2010

Curry, R. J., & Lu, Y. (2016). Synaptic Inhibition in Avian Interaural Level Difference Sound Localizing Neurons [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *eneuro*, *3*(6), Nov-Dec. https://doi.org/https://dx.doi.org/10.1523/ENEURO.0309-16.2016

de Siqueira, D. V. F., Strazza, P. S., Jr., Benites, N. M., & Leão, R. M. (2022). Salicylate activates KATP channels and reduces spontaneous firing in glycinergic cartwheel neurons in the dorsal cochlear nucleus of rats [Article]. *European Journal of Pharmacology*, *926*, Article 175026. https://doi.org/10.1016/j.ejphar.2022.175026

Dergacheva, O., Fleury-Curado, T., Polotsky, V. Y., Kay, M., Jain, V., & Mendelowitz, D. (2020). GABA and glycine neurons from the ventral medullary region inhibit hypoglossal motoneurons. *Sleep*, *43*(6). https://doi.org/10.1093/sleep/zsz301

Dyavanapalli, J., Byrne, P., & Mendelowitz, D. (2013). Activation of D2-like dopamine receptors inhibits GABA and glycinergic neurotransmission to pre-motor cardiac vagal neurons in the nucleus ambiguus [Article]. *Neuroscience*, *247*, 213-226. https://doi.org/10.1016/j.neuroscience.2013.05.039

Fischl, M. J., & Burger, R. M. (2014). Glycinergic transmission modulates GABAergic inhibition in the avian auditory pathway [Article]. *Frontiers in Neural Circuits*, *8*(MAR), Article 19. https://doi.org/10.3389/fncir.2014.00019

Frenkel, L., Muraro, N. I., Beltrán González, A. N., Marcora, M. S., Bernabó, G., Hermann-Luibl, C., Romero, J. I., Helfrich-Förster, C., Castaño, E. M., Marino-Busjle, C., Calvo, D. J., & Ceriani, M. F. (2017). Organization of Circadian Behavior Relies on Glycinergic Transmission [Article]. *Cell Reports*, *19*(1), 72-85. https://doi.org/10.1016/j.celrep.2017.03.034

Friauf, E., Aragon, C., Lohrke, S., Westenfelder, B., & Zafra, F. (1999). Developmental expression of the glycine transporter GLYT2 in the auditory system of rats suggests involvement in synapse maturation [Research Support, Non-U.S. Gov't]. *Journal of Comparative Neurology*, *412*(1), 17-37. https://doi.org/10.1002/(SICI)1096-9861(19990913)412:1%3C17::AID-CNE2%3E3.0.CO;2-E

Hoon, M., Soykan, T., Falkenburger, B., Hammer, M., Patrizi, A., Schmidt, K. F., Sassoe-Pognetto, M., Lowel, S., Moser, T., Taschenberger, H., Brose, N., & Varoqueaux, F. (2011). Neuroligin-4 is localized to glycinergic postsynapses and

regulates inhibition in the retina. *Proceedings of the National Academy of Sciences of the United States of America*, *108(7)*, 3053-3058. https://doi.org/10.1073/pnas.1006946108

Ito, T., Inoue, K., & Takada, M. (2015). Distribution of glutamatergic, GABAergic, and glycinergic neurons in the auditory pathways of macaque monkeys. *Neuroscience*, *310*, 128-151. https://doi.org/https://doi.org/10.1016/j.neuroscience.2015.09.041

Jaiswal, S. J., Buls Wollman, L., Harrison, C. M., Pilarski, J. Q., & Fregosi, R. F. (2016). Developmental nicotine exposure enhances inhibitory synaptic transmission in motor neurons and interneurons critical for normal breathing [Article]. *Developmental Neurobiology*, *76*(3), 337-354. https://doi.org/10.1002/dneu.22318

Kawai, N., Sakai, N., Okuro, M., Karakawa, S., Tsuneyoshi, Y., Kawasaki, N., Takeda, T., Bannai, M., & Nishino, S. (2015). The Sleep-Promoting and Hypothermic Effects of Glycine are Mediated by NMDA Receptors in the Suprachiasmatic Nucleus. *Neuropsychopharmacology*, *40*(6), 1405-1416. https://doi.org/10.1038/npp.2014.326

Kotak, V. C., Korada, S., Schwartz, I. R., & Sanes, D. H. (1998). A developmental shift from GABAergic to glycinergic transmission in the central auditory system [Article]. *Journal of Neuroscience*, *18*(12), 4646-4655. https://doi.org/10.1523/jneurosci.18-12-04646.1998

Kramer, F., Griesemer, D., Bakker, D., Brill, S., Franke, J., Frotscher, E., & Friauf, E. (2014). Inhibitory glycinergic neurotransmission in the mammalian auditory brainstem upon prolonged stimulation: Short-term plasticity and synaptic reliability [Article]. *Frontiers in Neural Circuits*, *8*(MAR), Article 14. https://doi.org/10.3389/fncir.2014.00014

Leao, R. N., Oieskevich, S., Sun, H., Bautista, M., Fyffe, R. E. W., & Walmsley, B. (2004). Differences in Glycinergic mIPSCs in the Auditory Brain Stem of Normal and Congenitally Deaf Neonatal Mice [Article]. *Journal of Neurophysiology*, *91*(2), 1006-1012. https://doi.org/10.1152/jn.00771.2003

Li, J., Nie, H., Bian, W., Dave, V., Janak, P. H., & Ye, J.-H. (2012). Microinjection of Glycine into the Ventral Tegmental Area Selectively Decreases Ethanol Consumption. *Journal of Pharmacology and Experimental Therapeutics*, *341*(1), 196. https://doi.org/10.1124/jpet.111.190058

Li, Y. Q., Takada, M., Kaneko, T., & Mizuno, N. (1998). Distribution of GABAergic and glycinergic premotor neurons projecting to the facial and hypoglossal nuclei in the rat [Research Support, Non-U.S. Gov't]. *Journal of Comparative Neurology*, *378*(2), 283-294. https://doi.org/10.1002/(SICI)1096-9861(19970210)378:2%3C283::AID-CNE10%3E3.0.CO;2-T

Mayadali, U. S., Fleuriet, J., Mustari, M., Straka, H., & Horn, A. K. E. (2021). Transmitter and ion channel profiles of neurons in the primate abducens and trochlear nuclei. *Brain Structure and Function*, *226(7)*, 2125-2151. https://doi.org/10.1007/s00429-021-02315-7

Mayer, F., Albrecht, O., Dondzillo, A., & Klug, A. (2014). Glycinergic inhibition to the medial nucleus of the trapezoid body shows prominent facilitation and can sustain high levels of ongoing activity. *Journal of Neurophysiology*, *112(11)*, 2901-2915. https://doi.org/https://doi-org.ezproxy.library.sydney.edu.au/10.1152/jn.00864.2013

McCracken, L. M., Lowes, D. C., Salling, M. C., Carreau-Vollmer, C., Odean, N. N., Blednov, Y. A., Betz, H., Harris, R. A., & Harrison, N. L. (2017). Glycine receptor α3 and α2 subunits mediate tonic and exogenous agonist-induced currents in forebrain. *Proc Natl Acad Sci U S A*, *114*(34), E7179-e7186. https://doi.org/10.1073/pnas.1703839114

Mitoh, Y., Funahashi, M., Kobashi, M., & Matsuo, R. (2004). Excitatory and inhibitory postsynaptic currents of the superior salivatory nucleus innervating the salivary glands and tongue in the rat [Article]. *Brain Research*, *999*(1), 62-72. https://doi.org/10.1016/j.brainres.2003.11.053

Morales, F. R., Silveira, V., Damian, A., Higgie, R., & Pose, I. (2011). The possible additional role of the cystic fibrosis transmembrane regulator to motoneuron inhibition produced by glycine effects. *Neuroscience*, *177*, 138-147. https://doi.org/10.1016/j.neuroscience.2010.12.034

Müller, N. I. C., Paulußen, I., Hofmann, L. N., Fisch, J. O., Singh, A., & Friauf, E. (2022). Development of synaptic fidelity and action potential robustness at an inhibitory sound localization circuit: effects of otoferlin-related deafness [Article]. *Journal of Physiology*, *600*(10), 2461-2497. https://doi.org/10.1113/JP280403

Nakamura, S., Inoue, T., Nakajima, K., Moritani, M., Nakayama, K., Tokita, K., Yoshida, A., & Maki, K. (2008). Synaptic transmission from the supratrigeminal region to jaw-closing and jaw-opening motoneurons in developing rats

[Research Support, Non-U.S. Gov't]. *Journal of Neurophysiology*, *100*(4), 1885-1896. https://doi.org/https://dx.doi.org/10.1152/jn.01145.2007

Neff, R. A., Wang, J., Baxi, S., Evans, C., & Mendelowitz, D. (2003). Respiratory sinus arrhythmia: Endogenous activation of nicotinic receptors mediates respiratory modulation of brainstem cardioinhibitory parasympathetic neurons [Article]. *Circulation Research*, *93*(6), 565-572. https://doi.org/10.1161/01.RES.0000090361.45027.5B

Noguchi, T., Nakamura, S., Nakayama, K., Mochizuki, A., Dantsuji, M., Ihara, Y., Takahashi, K., & Inoue, T. (2022). Developmental changes in GABAergic and glycinergic synaptic transmission to rat motoneurons innervating jaw-closing and jaw-opening muscles [Article]. *Brain Research*, *1777*, Article 147753. https://doi.org/10.1016/j.brainres.2021.147753

O'Brien, J. A., & Berger, A. J. (1999). Cotransmission of GABA and glycine to brain stem motoneurons [Research Support, U.S. Gov't, P.H.S.]. *Journal of Neurophysiology*, *82*(3), 1638-1641. https://doi.org/10.1152/jn.1999.82.3.1638

O'Brien, J. A., Sebe, J. Y., & Berger, A. J. (2004). GABA(B) modulation of GABA(A) and glycine receptor-mediated synaptic currents in hypoglossal motoneurons [Comparative Study Research Support, U.S. Gov't, P.H.S.]. *Respiratory Physiology & Neurobiology*, *141*(1), 35-45. https://doi.org/10.1016/j.resp.2004.03.009

Optican, L. M. (2008). The role of omnipause neurons: why glycine? [Research Support, N.I.H., Intramural]. *Progress in Brain Research*, *171*, 115-121. https://doi.org/10.1016/s0079-6123(08)00615-8

Rahman, J., Besser, S., Schnell, C., Eulenburg, V., Hirrlinger, J., Wojcik, S. M., & Hulsmann, S. (2015). Genetic ablation of VIAAT in glycinergic neurons causes a severe respiratory phenotype and perinatal death [Research Support, Non-U.S. Gov't]. *Brain Structure & Function*, *220*(5), 2835-2849. https://doi.org/https://dx.doi.org/10.1007/s00429-014-0829-2

Richardson, B. D., & Rossi, D. J. (2017). Recreational concentrations of alcohol enhance synaptic inhibition of cerebellar unipolar brush cells via pre- and postsynaptic mechanisms. *J Neurophysiol*, *118*(1), 267-279. https://doi.org/10.1152/jn.00963.2016

Russier, M., Kopysova, I. L., Ankri, N., Ferrand, N., & Debanne, D. (2002). GABA and glycine co-release optimizes functional inhibition in rat brainstem motoneurons in vitro [Research Support, Non-U.S. Gov't]. *Journal of Physiology*, *541*(Pt 1), 123-137. https://doi.org/10.1113%2Fjphysiol.2001.016063

Sebe, J. Y., Eggers, E. D., & Berger, A. J. (2003). Differential effects of ethanol on GABA(A) and glycine receptor-mediated synaptic currents in brain stem motoneurons [Research Support, U.S. Gov't, P.H.S.]. *Journal of Neurophysiology*, *90*(2), 870-875. https://doi.org/10.1152/jn.00119.2003

Sharp, D. B., Wang, X., & Mendelowitz, D. (2014). Dexmedetomidine decreases inhibitory but not excitatory neurotransmission to cardiac vagal neurons in the nucleus ambiguus [Article]. *Brain Research*, *1574*, 1-5. https://doi.org/10.1016/j.brainres.2014.06.010

Shino, M., Kaneko, R., Yanagawa, Y., Kawaguchi, Y., & Saito, Y. (2011). Electrophysiological characteristics of inhibitory neurons of the prepositus hypoglossi nucleus as analyzed in Venus-expressing transgenic rats [Research Support, Non-U.S. Gov't]. *Neuroscience*, *197*, 89-98. https://doi.org/https://dx.doi.org/10.1016/j.neuroscience.2011.09.017

Talman, W. T., Robertson, S. C., & Cassell, M. D. (1992). Mechanisms of cardiovascular responses to glycine injected into the dorsal vagal motor nucleus in rat [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. *Hypertension*, *19*(2 Suppl), II187-192. https://doi.org/10.1161/01.hyp.19.2_suppl.ii187

Torres Cadenas, L., Fischl, M. J., & Weisz, C. J. C. (2020). Synaptic Inhibition of Medial Olivocochlear Efferent Neurons by Neurons of the Medial Nucleus of the Trapezoid Body [Research Support, N.I.H., Intramural]. *Journal of Neuroscience*, *40*(3), 509-525. https://doi.org/https://dx.doi.org/10.1523/JNEUROSCI.1288-19.2019

Turecek, J., & Regehr, W. G. (2020). Cerebellar and vestibular nuclear synapses in the inferior olive have distinct release kinetics and neurotransmitters. *eLife*, *9*. https://doi.org/10.7554/eLife.61672

Valencia Garcia, S., Brischoux, F., Clément, O., Libourel, P.-A., Arthaud, S., Lazarus, M., Luppi, P.-H., & Fort, P. (2018). Ventromedial medulla inhibitory neuron inactivation induces REM sleep without atonia and REM sleep behavior disorder. *Nature Communications*, *9*(1), 504. https://doi.org/10.1038/s41467-017-02761-0

Wang, X., Piñol, R. A., Byrne, P., & Mendelowitz, D. (2014). Optogenetic stimulation of locus ceruleus neurons augments inhibitory transmission to parasympathetic cardiac vagal neurons via activation of brainstem α1 and β1 receptors [Article]. *Journal of Neuroscience*, *34*(18), 6182-6189. https://doi.org/10.1523/JNEUROSCI.5093-13.2014

Wollman, L. B., Levine, R. B., & Fregosi, R. F. (2018). Developmental nicotine exposure alters glycinergic neurotransmission to hypoglossal motoneurons in neonatal rats [Article]. *Journal of Neurophysiology*, *120*(3), 1135-1142. https://doi.org/10.1152/jn.00600.2017

Xing, H., Cui, N., Johnson, C. M., Faisthalab, Z., & Jiang, C. (2021). Dual synaptic inhibitions of brainstem neurons by GABA and glycine with impact on Rett syndrome [Article]. *Journal of Cellular Physiology*, *236*(5), 3615-3628. https://doi.org/10.1002/jcp.30098

Ye, J. H., Sokol, K. A., & Bhavsar, U. (2009). Glycine Receptors Contribute to Hypnosis Induced by Ethanol. *Alcoholism: Clinical and Experimental Research*, *33*(6), 1069-1074. https://doi.org/https://doi.org/10.1111/j.1530-0277.2009.00928.x

Ye, M., Hayar, A., Strotman, B., & Garcia-Rill, E. (2010). Cholinergic modulation of fast inhibitory and excitatory transmission to pedunculopontine thalamic projecting neurons [Research Support, N.I.H., Extramural]. *Journal of Neurophysiology*, *103*(5), 2417-2432. https://doi.org/https://dx.doi.org/10.1152/jn.01143.2009

Zhang, B., Gokce, O., Hale, W. D., Brose, N., & Sudhof, T. C. (2018). Autism-associated neuroligin-4 mutation selectively impairs glycinergic synaptic transmission in mouse brainstem synapses [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]. *Journal of Experimental Medicine*, *215*(6), 1543-1553. https://doi.org/https://dx.doi.org/10.1084/jem.20172162

Chapter 3: Glycinergic neurons in the periaqueductal grey are a novel population of inhibitory projection neurons involved in pain, anxiety and aversion

Glycinergic neurons in the periaqueductal grey are a novel population of inhibitory projection neurons involved in pain, anxiety and aversion

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[1] The submitted publication will include additional significant contributions from Rebecca Power and Karin Aubrey.

3.1 Abstract

The midbrain periaqueductal grey (PAG) is a critical integrative hub that modulates both sensory and emotional responses to pain. Despite its central role, the understanding of the underlying circuitry supporting its diverse functions remains incomplete. We have recently shown that glycine transporter-2 expressing neurons (PAG^{GlyT2}), concentrated in the ventrolateral PAG (vlPAG), bidirectionally modulate nociception. Here, we further characterise the distribution and connections of PAG^{GlyT2} neurons and examine whether their influence extends beyond nociceptive processing. Using GlyT2::Cre male and female mice, we selectively expressed fluorescent anterograde tracers, the genetically encoded calcium indicator GCaMP8s and chemogenetic DREADDs in PAG^{GlyT2} neurons. We demonstrated that these neurons are predominantly inhibitory projection neurons, with axonal terminals present in descending regions such as the rostral ventromedial medulla and locus coeruleus, as well as ascending regions including the paraventricular thalamus and lateral hypothalamus. Furthermore, we found that PAG^{GlyT2} neurons are engaged in response to both noxious and non-noxious stimuli and in addition to modulating nociception, they also modulate anxiety-like behaviours and aversion. Together these findings provide the first evidence that PAG^{GlyT2} neurons are inhibitory projection neurons that modulate both pain sensation and affective responses, likely mediated through their projections to both descending and ascending regions. This discovery expands our understanding of PAG circuitry and reveals a novel pathway by which nociceptive signals may be integrated with affective states, offering new insights into how pain is processed in the brain.

3.2 Introduction

The midbrain periaqueductal grey (PAG) is a central integrative structure that coordinates sensory, emotional, and cognitive information to shape pain responses in a context-dependent manner. It achieves this through extensive connections with both sensory and affective brain regions, allowing it to regulate a wide repertoire of behaviours, including passive and active defensive responses, autonomic control, aversion, and sleep (Arico et al., 2017; Ho et al., 2018; Keay & Bandler, 2015; Tovote et al., 2016; Weber et al., 2018). Anatomically, the PAG is organised into four longitudinal columns which are defined by distinct functional roles and projection targets (Keay & Bandler, 2015). The ventrolateral column of the PAG (vlPAG) is a key hub for opioid mediated analgesia and the descending analgesic pathways, composed primarily of excitatory projections under the control of inhibitory interneurons (Lau & Vaughan, 2014; Vaughan et al., 1997; Winters et al., 2022). The vlPAG has also been implicated in many other behaviours including defensive responses, fear, predatory hunting, anxiety, depression, cardiovascular function, respiratory regulation, sleep, and bladder control (see Zhang et al., 2024 for a comprehensive review).

There are many different neuronal subtypes in the vlPAG which are believed to contribute to its integration of different behaviours (Vaughn et al., 2022). This suggests that there are different functional units present within this subpopulation of neurons that allow the vlPAG to modulate behaviours in a contextually appropriate manner. Understanding of how these different functional units in the vlPAG is organised to control all these behaviours and how different neuronal cell types and their microcircuits contribute to this is still largely unknown. This is particularly relevant in the context of chronic pain, where vlPAG dysfunction contributes not only to altered nociception but also to emotional and cognitive comorbidities (Deng et al., 2023; Lee et al., 2023; Samineni et al., 2019; Yang et al., 2022).

Glycinergic neurons, defined by expression of the glycine transporter GlyT2, are highly expressed in the spinal cord where they modulate pain signalling, however their supraspinal roles are only beginning to be explored (Fenech et al., 2024; Zafra et al., 2016; Zeilhofer, 2005; Zeilhofer et al., 2005) A small population of GlyT2-expressing neurons is concentrated in the vlPAG (PAG^{GlyT2}) and we previously demonstrated that they bidirectionally modulate thermal nociceptive responses and locomotor activity in male mice (Assareh et al., 2023; Rampon et al.,

1996; Zeilhofer et al., 2005). Here, we characterise the distribution and abundance of PAG^{GlyT2} neurons in the vlPAG and use circuit tracing, *in vivo* calcium imaging and employ chemogenetic strategies and behavioural assays to map their connectivity and activity. We demonstrate that unlike the majority of inhibitory PAG interneurons, PAG^{GlyT2} neurons are inhibitory projection neurons that can sense and modulate both sensory and affective dimensions of pain. These findings expand our understanding of vlPAG neuroanatomy and reveal a previously unrecognised signalling pathway that contributes to the integration and modulation of pain.

3.3 Methods

Animals

All experiments were conducted in accordance with the Australian Code for the care and use of animals for scientific purposes and approved by the University of Sydney Animal Ethics Committee (Protocol: 2023/2295). Adult male and female GlyT2::Cre (a gift from H.U. Zeilhofer; Foster et al., 2015) or GlyT2::cre x Ai14 mice (Jackson Laboratory; B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, Strain #: 007914) were bred and housed in the Kearns Animal Facility in ventilated cages with a maximum of four mice per cage. 12/12h light/dark cycle (23°C, 70% humidity) were maintained and food and water provided ad libitum. Cages were enriched with a house igloo, toilet paper roll, tissues for nesting and wooden blocks. All behavioural experiments were conducted during the light cycle between 9AM and 5PM under dim white light (30 +/- 10 Lux).

Stereotaxic surgery

Brain injections were carried out in a stereotaxic frame (Kopf Instruments) in adult mice (8-12 weeks old) under isoflurane anaesthesia (1-3% isoflurane in 1L/min O2). Pain relief was provided at the incision site (0.25% bupivacaine s.c) and viral vectors were injected with a nanovolume needle (200-300 nL (vlPAG); SEG Syringe 1mL, 0.63 mm OD, part #00500) using a motorized injector (50 nL/min, UMP3T-2 with SMARTouch, WPI) or microinjection pipet (Drummond Nanoject II) using glass needles with an external diameter of 60 µm (20-50 nL for LH and PVT injections). The following coordinates were used:

- vlPAG- from Bregma, AP: -4.65-4.7 mm, ML: ±0.35 mm, DV: -3.00 mm or AP: -4.65 mm, ML: ±0.85 mm, DV: -2.54 mm on an 8-degree angle (which reduced loss of vector in the aqueduct and improved bilateral injection hit rate).
- LH- from Bregma, AP: -1.4 mm, ML: -1.1 mm, DV: -5 mm
- PVT- from Bregma, AP: -1.4 mm, ML: -1.1 mm, DV: -5 mm

Bilateral injections into the vlPAG were made for all chemogenetic experiments and unilateral for circuit tracing and fibre photometry. After injection, the needle was kept in place for five minutes, retracted 0.1mm and left for a further five minutes before complete withdrawal.

For fibre photometry studies, following viral injection into the left or right vlPAG (counterbalanced across animals), a fibreoptic implant (200 µm diameter, 0.39 NA, RWD Science) was implanted at vlPAG injection site and secured with the help of two small skull screws and Superbond C & B (Sun Medical) and Vertex self-Curing (Vertex Dental) applied to fix the fibre in place. Monocryl sutures 6-0 were used to close the wound and pain relief was administered (buprenorphine, 0.05-0.1mg/g mice, s.c.). Following surgery, the mice were individually housed for recovery (two days) before being returned to their home cage. Experiments commenced two (fiber photometry) or three weeks later (behaviour, electrophysiology or histology). For the chemogenetic study, animals were randomly allocated into a control (mCherry), hM3Dq or hM4Di group where they were injected (i.p.) with either vehicle (10% DMSO and saline) or CNO (3mg/kg hM3D(Gq) or 5mg/kg hM4D(Gi)/mCherry control) on alternate behavioural testing days (minimum two-day interval).

Nociceptive tests

Nociceptive tests were all carried out on a single day with a minimum of 5 minutes rest periods between tests except if noted (Anderson et al., 2014; Assareh et al., 2023; François et al., 2017; Mitchell et al., 2021).

von Frey

To assess mechanical hypersensitivity, we used the simplified up-down method (SUDO) using Von Frey filaments (Bonin et al., 2014). Animals were placed in elevated Perspex enclosures with wire mesh bases and given 30 minutes to acclimatise to the testing environment. Testing began with a 0.95 g von Frey hair in which it was pressed perpendicularly against the left hind paw and held for approximately two seconds. If the animal sharply withdrew their paw, licked their paw, or flinched upon removal of the von Frey filament, a withdrawal response was noted. The von Frey test was repeated four times on the injected paw with at least a two-minute interval between tests. If a positive withdrawal response was observed, the subsequent test used a lighter filament whereas if no withdrawal response was seen the subsequent test used the next heavier filament. The final paw withdrawal threshold was calculated as the lightest force required to produce a withdrawal response. For the fibre photometry experiments, the 0.16g,

2g and 6g von Frey filaments were pressed against the left hind paw in ascending order, with five trials of each filament with a two-minute interval in between.

Acetone test

To assess cold allodynia, we used the acetone test (Yoon et al., 1994). Animals remained in the elevated Perspex enclosures with wire mesh bases, and 20µL of acetone was sprayed on the centre of the left hind paw through the wire grid. The number of licks was recorded over a one-minute period. The test was repeated once on the same paw with at least five minutes between tests. The final value used for statistical analysis was the average of the number of licks between the two tests.

Hotplate test

To assess noxious heat sensitivity, we used the hotplate test. Each animal was placed on a 50℃ hotplate and latency to show either a licking or jumping nocifensive response was recorded. Once observed, the animal was immediately removed from the hotplate and returned to their homecage. Each animal was tested for a minimum of three trials with a fourth and fifth trial added if the results were not within four seconds of each other (Espejo et al., 1994). For the fibre photometry experiments, noxious heat sensitivity was assessed with the dynamic hotplate test in which it started at 31.4℃ and increased by 3.4℃/min to finally reach 50℃ where they remained for 30 seconds before removed from the apparatus. Hotplate experiments for the fibre photometry experiments were completed on a separate testing day to von Frey and acetone.

Open Field test

Locomotor activity was measured using the open field test. One hour after i.p. injection of either vehicle or CNO and acclimatisation to the testing room, the mice were individually placed in an enclosed open-top arena (50 x 50 x 50 cm). An overhead camera recorded their behaviour for a 20-minute period. This period allowed for the monitoring of locomotion and potential anxiety-like behaviours (Bailey & Crawley, 2009). Between animals, 20% ethanol was used to clean the apparatus to remove odour cues. The number of faecal boli, supported rearing

responses and jumping responses was scored manually using Chronotate (Philipsberg et al., 2023) while the total time mobile (s), time in the centre zone (s) and number of entries into the centre zone was obtained through ANY-Maze video tracking software (Version 7; Stoelting Co.). The centre zone was classified as the middle 25cm x 25cm zone.

Light-Dark test

The light-dark box test was used to test anxiety-like behaviours (Bourin & Hascoët, 2003). One hour after i.p. injection of either vehicle or CNO and acclimatisation to the testing room, the mice were individually placed in the light-dark box (dark chamber: 20 cm wide x 18 cm x 30 cm high; light chamber (60 ± 10 lux): 28 cm wide x 27 cm long x 30 cm high). The mice were initially placed in the light chamber facing the entry to the dark chamber and behaviour monitored with an overhead camera for five minutes. Between animals, 20% ethanol was used to clean the apparatus to remove odour cues. The following measures were obtained following ANY-Maze video tracking software analysis: number of entries into the light zone, time in the light zone (s) and latency to enter the dark zone (s).

Elevated Plus Maze

The elevated plus maze test was used in conjunction with the light-dark box to test anxiety-like behaviours on a subset of animals (Riebe & Wotjak, 2012). One hour after i.p. injection of either vehicle or CNO and acclimatisation to the testing room, the animals were individually placed in the centre of the elevated plus maze facing a closed arm (two 60 cm arms, each 5cm wide, one enclosed by 15 cm walls, 40 cm elevated off the ground). An overhead camera recorded their behaviour for a five-minute period. Between animals, 20% ethanol was used to clean the apparatus to remove odour cues. The time spent (s) in the open and closed arms was obtained using ANY-Maze video tracking software.

Conditioned Place Aversion

The conditioned place aversion test was used to evaluate if activation or inhibition of PAG^{GlyT2} neurons is aversive. The apparatus was visually and tactile differentiable between the two zones with one side with smooth blue floors and walls with bright yellow circle stickers on the walls

while the other side had a bright silicone pink floor with smooth blue walls (Scicluna et al., 2024). Mice were placed in a non-biased two-chamber CPA arena and given free access for a 30-minute period for three preconditioning training days. Time spent in either side was averaged across the last two days such that no animals spent less than 30% of their time in the one zone. Neither zone was found to have an inherent biased preference. Two days later, conditioning was carried out using a biased experimental design for six training days. CNO was exclusively paired with zones that had 60% or greater time spent in the pre-conditioning stage. Vehicle was paired to the other zone. Mice that spent between 40-60% of their time in either zone during preconditioning were randomly allocated and counterbalanced across groups. Postconditioning test days: two and eight days later mice were given free access to both zones and the time spent in each zone was recorded. Time spent is presented as a percentage of overall test duration (time animal spent inside the box). A lid was placed on top of the apparatus to ensure no climbing behaviour during the conditioning and post conditioning days. Automated or manual behavioural tracking of the centre point of the animal was used to measure total time in either side for the pre-conditioning days (AnyMAZE) as the lid obscured automatic tracking in some cases. Between animals, 20% ethanol was used to clean the apparatus to remove odour cues. To show a complete picture of the conditioned place aversion test, time spent in the CNO-paired zone as well as difference scores between treatment and pre and post conditioning (Yates, 2023). Difference scores were calculated as time difference between vehicle and CNO paired zones in the pre and post conditioning stages (CNO-vehicle) and percentage of time difference between CNO-paired zone for the pre and post conditioning stages (post-pre).

Fibre photometry

Neuronal calcium activity was recorded via with the RWD fibre photometry system (R821 /FR-21Tricolor Multichannel Fiber Photometry System). A 410nm isosbestic signal was recorded to account for motion artefacts and the 470nm signal was used to detect GCaMP activity (30Hz, power = 20-30mW). Z-scores were calculated around events during behavioural experiments verified with one to two cameras during behavioural recordings using RWD software. Peak z-scores or area under the curve (AUC) was calculated before or after events using RWD software.

Aversive tone and footshock

For the fibre photometry experiments, aversive tones and foot shocks were used to assess non-noxious aversion and graded pain, respectively. Animals were placed in a footshock chamber within a clear tubular enclosure. After a five-minute acclimatization period, a buzzer was used to deliver a tone (5 kHz, 80 dB, 1 sec tone every 2 minutes x 5 trials; Assareh et al., 2025). After a two-minute recovery period, a foot shock stimulus of increasing intensity was presented to the animal (0.05mA, 0.1mA, 0.2mA and 0.4mA x 5 trials; two-minute interval between trial).

Tailgrab and claps

Tailgrab is considered a stressful stimulus (Hurst & West, 2010) and clap is an aversive sound stimulus. Animals were placed in their home cage. After a five-minute acclimatization period, the researcher picked up the animal by the tail (tail grabs; 5 trials, with two-minute interval between each trail). Following a 5-minute rest period, the researcher then delivered a single loud clap near the outer edge of the home cage (5 trials, with two-minute interval between each trial).

Tissue processing

At the end of the experimental period, mice were deeply anaesthetized (3-5% isoflurane; 1L/min O_2 then injection of Lethobarb (200 mg/kg, i.p)) and transcardially perfused first with 0.9% saline containing 72.5 mM NaNO2 and 3 IU/ml heparin (Sigma-Aldrich) and then 4% paraformaldehyde (PFA) in 0.13 M PBS (pH 7.4). Brains were removed and postfixed overnight in the same fixative solution (4% PFA, 4°C). The tissue was then washed in PBS (pH 7.4) and dehydrated in 15% sucrose in PBS for 1-2 days, followed by 30% sucrose in PBS for 1-2 days and stored until cryosectioning.

Brains were sectioned using a sliding microtome (-20℃, Leica Microsystems, Leica 1080) and four series of 40 μm slices were collected and preserved in 0.1 M PBS containing 0.1% sodium azide at 4°C. The PAG slices from one series were mounted onto glass slides with Fluromount-G Mounting Medium (Invitrogen) and a glass coverslip. After drying, coverslip was sealed with nail polish.

Immunofluorescence and fluorescent *in situ* hybridisation

For circuit tracing experiments, slices from another series were selected and labelled with immunofluorescence. Slices were washed in PBS (3 x 10 minutes), then incubated in blocking media (1 hour at RT, 10% donkey serum, 0.3% Triton X detergent in PBS). Then, sections were then incubated in primary antibody overnight before being washed in PBS (3 x 30 minutes) and incubated with secondary antibody (2 hours at RT;10% donkey serum, 0.1% Triton X Detergent, 1:2000 DAPI antibody). Following a final wash in phosphate buffer (PB, 3 x 30 minutes), slices were mounted as previously described for imaging.

Fluorescent *in situ* hybridization was performed on 30-µm coronal sections using RNAscope technology (Advanced Cell Diagnostics Biotechne, USA), following the manufacturer's protocol. The GAD2, Slc6A5 and Cre probes were used.

Fluorescent and light microscopy

Slices were imaged across the rostro-caudal axis on a fluorescent microscope (Zeiss Axio M1) and localized to region according to Paxinos and Franklin (2001). As the PAG is very close to the cerebral aqueduct, some DREADDs injections were unilateral, likely due to loss of vector into the aqueduct. The behavioural data obtained from animals with confirmed uni- and bilateral expression of mCherry, hM3D and hM4D was compared and no significant differences were detected, so data was pooled (Supplementary Figure 1). Sparse labelling of neurons in the superior colliculus was occasionally noted near the injection tract.

A laser confocal microscope (Leica Stellaris 5) using 40× (NA 1.25) objective was employed to capture images of synaptophysin-fused EGFP and tdTomato. Stacks of 10-15 µm optical sections were acquired at a pixel resolution of 0.28 µm and a z-step 1 µm.

Electrophysiology

Four weeks after stereotaxic surgery to express the excitatory DREADD channel hM3Dq or light activated channel rhodopsin (ChR) in PAG^{GlyT2} neurons, mice were deeply anesthetized,

sacrificed and the brain was rapidly removed and place into ice-cold N-methyl-D-glucamine solution (NMDG) containing (in mM): 93 NMDG, 30 NaHCO3, 25 glucose, 5 N-acetyl-L-cysteine, 3 Na-pyruvate, 2.5 KCl, 1.2 NaH2PO4, 20 HEPES, 10 MgSO4, 0.5 CaCl2, 5 sodium ascorbate (300 mOsm), equilibrated with 95% O2-5% CO2. Then 280-mm-thick coronal brain slices of the midbrain were prepared with a vibratome (Leica VT1200S) in the same ice-cold NMDG solution. Slices were maintained in this solution for 10min at 34°C and then transferred into the artificial CSF (ACSF; in mM: 126 NaCl, 2.5 KCl, 1.2 NaH2PO4,1.2 MgCl2, 2.4 CaCl2, 11 glucose, and 25 NaHCO3, equilibrated with 95% O2 and 5% CO2) for 1 hour before being place in RT ACSF until use. For recording, slices were individually transferred to a chamber on an upright fluorescence microscope (Olympus BX51) and superfused continuously with ACSF (33°C, flow rate 2.5 ml min-1). PAG neurons were visualized with a 40x water-immersion objective using Dodt gradient contrast optics and mCherry fluorescence was detected using epifluorescent illumination. Whole-cell patch-clamp recordings were performed in the voltage-clamp configuration with a holding potential of -65 mV. Patch pipettes (3–4MV) were filled with an intracellular solution composed of (in mM): 130 K-gluconate, 0.5 EGTA, 10 HEPES, 10 Na2phosphocreatine, 5 MgATP, 0.4 NaGTP, and 0.1% biocytin, pH 7.3 with KOH (290–295mOsm). The liquid junction potential was not corrected. Glycine currents were isolated pharmacologically using specific receptor antagonists as required: AMPA/kainate receptor antagonist, 2,3Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; 5 mM), the NMDA receptor antagonist, d-2-Amino-5-phosphonopentanoic acid (D-AP5; 25 mM), the GABA receptor antagonist bicuculine (100mM) and glycine receptor antagonists strychnine (STY; 3 mM). All recordings were filtered (2- to 10-kHz low pass filter) with Multiclamp 700B amplifier (Molecular Device) and digitized at a sampling rate of 10 kHz with an A/D converter (NI USB-6251, National Instruments) and acquired and analysed using AxographX or Clampfit (Molecular Device). PAG^{GlyT2} neurons were stimulated using either CNO (1-10 mM) or 470nm (blue) light delivered through the 40x objective. To visualize cells filled with biocytin during electrophysiology recording, brain slices were fixed in 10% formalin solution (Sigma) and then incubated with streptavidin Alexa-647 (1:1000, Abcam). Slices were rinsed in PBS, mounted with PermaFluor (Epredia), and stored at 4°C.

Data analysis

All data was analysed using GraphPad Prism (version 10, GraphPad Software, Inc., La Jolla, CA) and presented as mean ± SEM. Significance, as indicated by an asterisk, was defined as $p < 0.05$ a priori. For all datasets, a Shapiro-Wilk test was performed to test normality. Statistical tests used throughout this study include paired and unpaired t-tests, and one- and two-way ANOVAs. When post-hoc testing was appropriate, following a significant ANOVA test, the Bonferroni correction was applied to all data. A detailed statistical summary can be found in (Supplementary Table 1). All behavioural test schematics were created with BioRender.

Table 1: Vectors and antibodies.

Name	Company	CAT#	Titre/concentration
Viral Vectors			
AAV1-phSyn1-FLEX-TdTomato-T2A-SypEGFP-WPRE	Addgene	51509	4.5×10^{12}
AAV5-hSyn-DIO-mCherry	Addgene	50459	8.4×10^{12}
AAV5-hSyn-DIO-hM3D(Gq)-mCherry	Addgene	44361	2.3×10^{13}
AAV5-hSyn-DIO-hM4D(Gi)-mCherry	Addgene	44362	2.5×10^{13}
AAV9-EF1adflox-hChR2(H134R)-EYFP-WPRE-HGHpA	Addgene	20298	2.1×10^{13}
AAV9-syn-FLEX-jGCamp8s-WPRE	Addgene	162377	2.7×10^{13}
AAVrg-EF1a-DIO-EYFP	Addgene	27056	1.3×10^{13}
AAV8-hSyn-mCherry	Addgene	114472	2.6×10^{13}
Primary antibodies			
Mouse anti-TPH	Oncogene	OP71L	1:250
Rabbit anti-TH	Merck	AB152	1:2000
Rabbit anti-cFos	Cell signalling Tech	2250T	1:500 (fluorescence) 1:2000 (colorimetric)
Rabbit anti-pPDH	Cell signalling Tech	37115S	1:500
Chicken anti-GFP	Aveslabs	GFP-1020	1:500
Goat anti-tdTomato	Mybiosource	MBS448092	1:500

Chicken anti-mCherry	Abcam	Ab205402	1:1000
Secondary antibodies			
Donkey anti-mouse Alexa 647	Abcam	Ab150111	1:500
Donkey anti- rabbit Alexa 647	Abcam	Ab150075	1:500
Donkey anti-rabbit Alexa 488	Abcam	Ab150073	1:1000
Goat anti-chicken Alexa 568	Abcam	Ab175477	1:500
Donkey anti-chicken Alexa 488	Jackson Immuno Res	703-545-155	1:500
Donkey anti-goat Alexa 555	Abcam	Ab150134	1:500

3.4 Results

PAG^{GlyT2} neurons are concentrated in the ventrolateral PAG and express GAD2

To determine the number and distribution of PAG^{GlyT2} neurons across the rostral-caudal axis of mice, we performed cell counting and fluorescent *in situ* hybridisation on coronal brain slices from GlyT2::Cre x Ai14 or GlyT2::Cre mice. Consistent with previous reports, we found that most PAG^{GlyT2} neurons are found in the vlPAG, with highest numbers in the caudal PAG (Figure 1A-C). mRNA probes specific for GlyT2 (Slc6a5) and the GABA synthesis enzyme GAD65 (GAD2) were colocalised in 84.6 ± 2 % of PAG^{GlyT2} neurons and PAG^{GlyT2} neurons made up 17.4 ± 4% of PAG^{GAD65} neurons in the vlPAG. Together, this indicates that PAG^{GlyT2} neurons are a minority subset of inhibitory GABAergic neurons in the vlPAG that are likely to co-release glycine with GABA (Figure 1D-E). Finally, we found that mRNA labelling of Cre was almost exclusively associated with GlyT2 labelled neurons (96.23 ± 1.447 %) supporting the validity of the GlyT2::Cre mouse line, however Cre was sparse and colocalised with <25% of PAG^{GlyT2} neurons (Supplementary Figure 2; Foster et al., 2015).

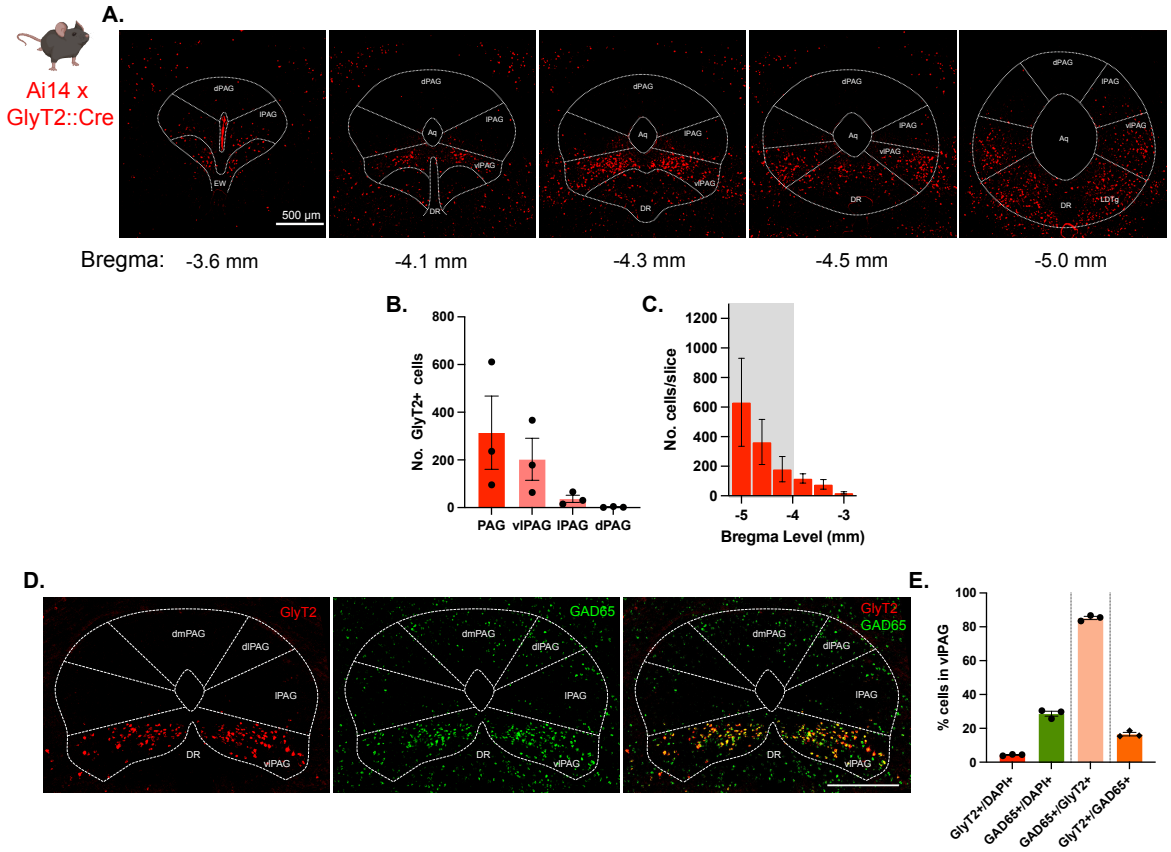


Figure 1: PAG^{GlyT2} neurons have restricted distribution in the vlPAG and are inhibitory neurons

(A) Distribution of GlyT2+ cells in the PAG. Rostral to caudal fluorescent image array (0.2-0.5µm apart) of mouse PAG with GlyT2 positive (red) cells amplified. Corresponding Bregma levels are indicated below each image. Scale Bar = 500µm. Widefield images shown are 4x4 tiled z-stacks (max intensity projection), taken with a 20x objective. Boundaries and abbreviations extrapolated from Paxinos and Franklin (2001). (B) Bar graph representing the average number of GlyT2+ cells within each PAG sub column (n=3 animals; mean +/- SEM). (C) Bar graph representing average number GlyT2+ cells (in 400 µm bins) within the caudal to rostral mouse PAG (grey) based on bregma levels (n=3 animals; mean +/- SEM). (D-E) Distribution and quantification of GlyT2+ and GAD65+ mRNA in the vlPAG using fluorescent *in situ* hybridisation (FISH). Dorsal periaqueductal gray (dPAG), lateral periaqueductal gray (lPAG), ventral lateral periaqueductal gray (vlPAG), laterodorsal tegmental nucleus (LDTg), dorsal raphe (DR), Edinger-Westphal nucleus (EW).

Activation of PAG^{GlyT2} neurons is not sensed by many neighbouring vlPAG neurons

Most vlPAG neurons that make local connections are inhibitory interneurons (Winters et al., 2022). To test the hypothesis that PAG^{GlyT2} neurons make strong local connections, we recorded spontaneous IPSCs from PAG^{GlyT2} -negative neurons in the vlPAG of GlyT2::Cre mice in which PAG^{GlyT2} neurons selectively expressed the light activated channel ChR2 or the excitatory DREADD channel hM3Dq (Figure 2A). The amplitude and frequency of sIPSCs were strongly inhibited by the GABA receptor antagonist bicuculline, with the remaining residual current inhibited by the glycine receptor antagonist strychnine. Therefore, both GABA and glycine contribute to sIPSCs in the vlPAG (Figure 2B). Surprisingly, sIPSCs amplitude and frequency did not change in 32/33 (14 ChR, 19 hM3D) PAG^{GlyT2} -negative neurons recorded in response to specific stimulation of PAG^{GlyT2} neurons (either 470 nm (blue) light (n=14) or CNO (n=19)). However, in one recording from a slice containing hM3D expressing PAG^{GlyT2} neurons, CNO stimulated a burst of GABA and glycine mediated sIPSCs. These data provide light evidence that PAG^{GlyT2} neurons can release GABA and glycine within the vlPAG and contribute to local inhibitory signalling but suggest that they do not make extensive functional synaptic connections within the vlPAG.

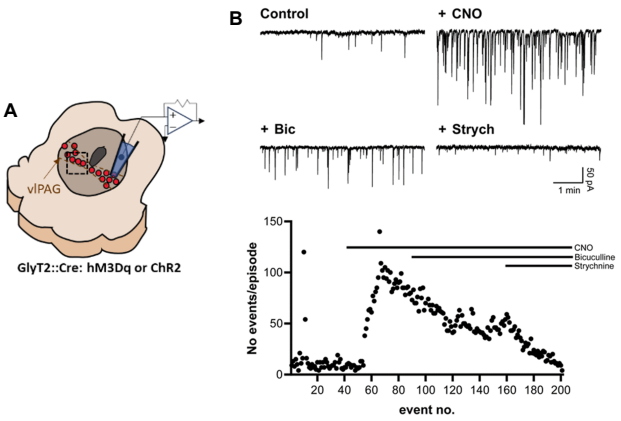


Figure 2: Inhibitory post synaptic currents (IPSCs) are rarely detected in undefined vlPAG neurons following selective activation of PAG^{GlyT2} neurons. (A) Experimental set up showing electrophysiology recording in whole-cell voltage-clamp from undefined vlPAG neurons in midbrain slices containing PAG^{GlyT2} neurons expressing either hM3D or ChR2. (B) Spontaneous IPSC recorded before and after selective activation of PAG^{GlyT2} neurons with DREADD agonist CNO. The GABA- and glycine receptor contributions to the recorded current is indicated by its sensitivity to selective GABAA and glycine receptor antagonists bicuculline and strychnine, respectively.

PAG^{GlyT2} neurons have projections to descending and ascending brain regions

To map the circuit connections of PAG^{GlyT2} neurons, we expressed AAV1-phSyn-FLEX-tdTom-T2A-SypEGFP in PAG^{GlyT2} neurons and imaged serial slices throughout the mouse brain. This vector expresses tdTomato in the soma, dendrites and axons of PAG^{GlyT2} neurons and GFP in axon terminals that express the presynaptic marker synaptophysin, allowing for an anatomical assessment of PAG^{GlyT2} neuronal circuits and where they are making synapses (Figure 3A). In addition to the vlPAG, tdTomato^{+} axons and terminals were detected within a variety of descending and ascending brain nuclei in the hypothalamus and midline thalamus (Figure 3B-D). All regions identified have been previously reported to be targeted by vlPAG projection neurons (Cameron, Khan, Westlund, Cliffer, et al., 1995; Cameron, Khan, Westlund, & Willis, 1995). Together, these experiments suggest that PAG^{GlyT2} neurons are inhibitory projection neurons which make direct synaptic connections to both descending and ascending regions. In support of this proposition, we found that stereotaxic injection of small volumes of Cre-dependent AAV-retrograde traces into hypothalamus and midline thalamus regions with visible PAG^{GlyT2} projections, results in labelling of PAG^{GlyT2} neurons in the caudal PAG, particularly concentrated around bregma level -4.5 to -4.7mm (Figure 3E-F).

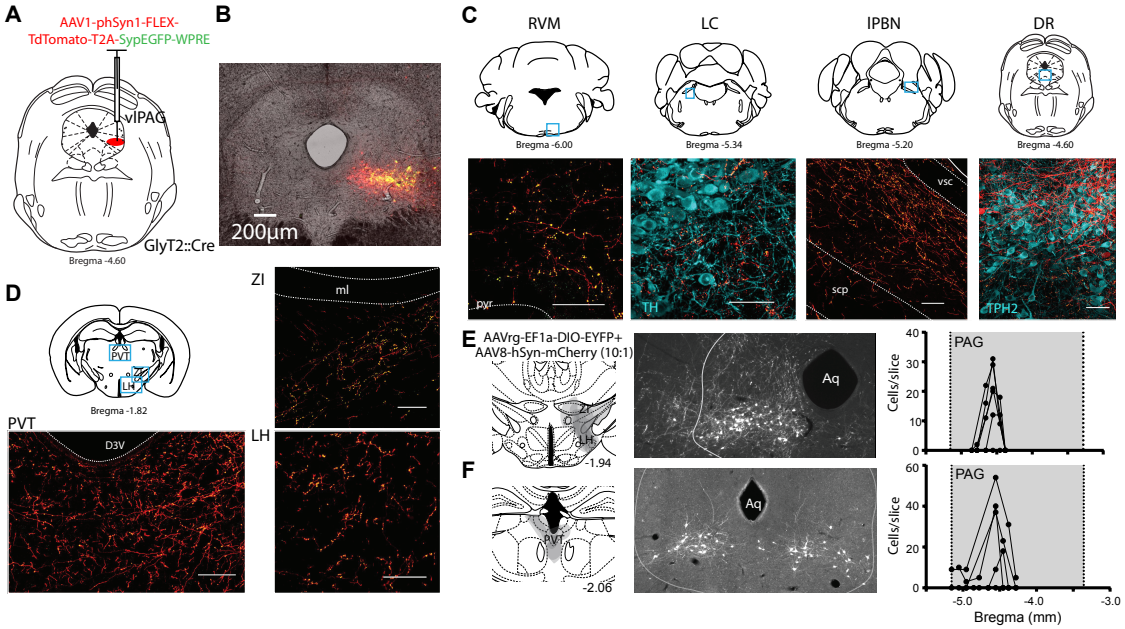


Figure 3: PAG^{GlyT2} neurons are projection neurons. (A) Schematic illustrating unilateral injection of AAV1-phSyn-FLEX-tdTom-T2A-SypEGFP into GlyT2::Cre mice. (B-D) Widespread PAG^{GlyT2} projections and terminals were detected in the midbrain (PAG, DR), brainstem (RVM), pons (lPBN, LC), thalamus (PVT) and hypothalamus (LH, ZI). Scale bars = 50 μm. (n=3). (E-F) Cre dependent retrograde trace injected into the projection regions in the hypothalamus (n=5) or thalamus (n=6) labelled subsets of PAG^{GlyT2} neurons in vlPAG (grey box), with a peak bregma ~-4.6 mm in both cases. RVM: rostral ventromedial medulla, LC: locus coeruleus, lPBN: lateral parabrachial nucleus, DR: dorsal raphe, PVT: paraventricular thalamus, ZI: zona incerta, LH: lateral hypothalamus, D3V: 3rd ventricle, ml: medial lemniscus, pyr: pyramidal tract, scp: superior cerebellar peduncle, vsc: ventral spinocerebellar tract, Aq: cerebral aqueduct, TH: tyrosine hydroxylase, TPH2: tryptophan hydroxylase 2.

In vivo functional activity of PAG^{GlyT2} neurons reveal they are responsive to both nociceptive and aversive stimuli

We have previously demonstrated that chemogenetic activation of PAG^{GlyT2} neurons can alter nociceptive responses in mice following noxious heat and cold stimuli (Assareh et al., 2023), however the activity of PAG^{GlyT2} neurons in response to external stimuli has never been assessed. To achieve this, we expressed the genetically encoded calcium indicator (jGCaMP8s) in PAG^{GlyT2} neurons and used *in vivo* fibre photometry to directly measure changes in their activity (Figure 4A, Supplementary Figure 3; Zhang et al., 2023). No change in PAG^{GlyT2} calcium signal was detected in response to increasing mechanical stimuli with von Frey hairs (0.16 – 6g; Figure 4B), but was increased in response to a range of nociceptive stimuli including

hind paw application of acetone (cold stimuli; Figure 4C), during rearing and jumping responses in a dynamic thermal plate assay (Figure 4D-E; Kimmey et al., 2025) and in response to electric shock stimuli of increasing intensity (0.05 mA, 0.1 mA, 0.2 mA and 0.4 mA; Figure 4F). As the vlPAG is known to contribute to a variety of functions in addition to nociception, including fear and defensive behaviour (Arico et al., 2017; Keay & Bandler, 2015; Tovote et al., 2016), we also measured PAG^{GlyT2} neuronal activity following different aversive non-noxious stimuli (auditory tone, clap and tail grab). We found significant increases in calcium signal in response to loud auditory tone (Figure 4G), sudden clap (Figure 4H) and tail grab (Figure 4I). Overall, these data demonstrate that PAG^{GlyT2} neurons directly respond to noxious and non-noxious stimuli. Our finding that their response to increasingly strong foot shock is scaled further suggests that PAG^{GlyT2} activity may signal pain intensity. In addition, we discovered that PAG^{GlyT2} neuronal activity increases during a range of behaviours that are not associated with a noxious stimulus including rearing, loud noises and tail grab. Together this suggests that PAG^{GlyT2} neurons may have the capacity to modulate other vlPAG mediated behaviours.

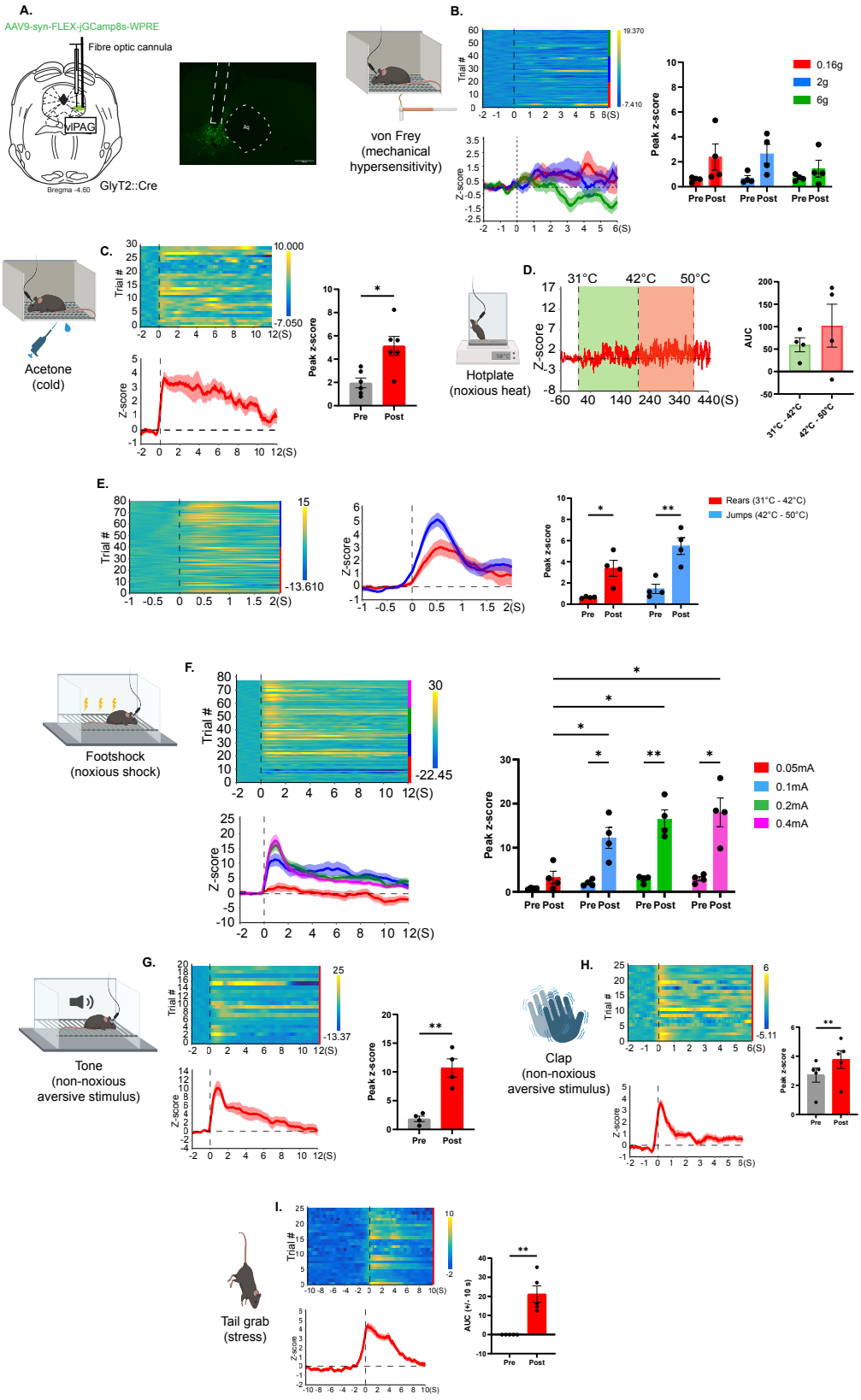


Figure 4: *in vivo* fibre photometry shows that PAG^{GlyT2} neurons are involved with nociception, aversion and stress. (A) Schematic and representative image showing unilateral

stereotaxic injection of cre-dependent calcium activity dependent viral vector into the vlPAG of GlyT2::Cre male mice, targeting PAG^{GlyT2} neurons (B) Average trace and heatmap of z-score during the 0.16g, 2g and 6g von Frey stimulus with quantification of peak z-score showing no indication of increased activity following either stimulus. (C) Average trace and heatmap of z-score during the acetone test with quantification of peak z-score showing increased activity following acetone stimulus. (D) Average trace of z-score during the dynamic hotplate assay test with quantification of the area under the curve (AUC) showing no significant increase in activity between the non-noxious and noxious phase of the test. (E) Average trace and heatmap of z-score during rearing responses (during the non-noxious 31-42 degree phase) and jumping responses (during the noxious 42-50deg phase) in the hotplate test with quantification of peak z-score showing increased activity following both responses. (F) Average trace and heatmap of z-score during an increasing foot shock stimulus with quantification of peak z-score of 0.05mA, 0.1mA, 0.2mA and 0.4mA, showing increased activity following 0.1mA, 0.2mA and 0.4mA shocks and compared to 0.05mA shocks. (G) Average trace and heatmap of z-score during an aversive tone with quantification of peak z-score showing increased activity following the tone. (H) Average trace and heatmap of z-score during an aversive clap with quantification of peak z-score showing increased activity following the clap. (I) Average trace and heatmap of z-score during a tail grab with quantification of AUC showing increased activity following the grab. Five trials per stimulus. Individual animals indicated (n= 3-5; mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Chemogenetic modulation of PAG^{GlyT2} neurons reveal that they alter anxiety-like behaviours in addition to nociceptive responses

The possibility that PAG^{GlyT2} neurons modulate behaviours beyond nociception was also suggested by our previous data, as we found they modulate jumping behaviour in the open field test (Assareh et al., 2023). To investigate this further, we selectively expressed DREADDs, hM3Dq, hM4Di or control (mCherry), in PAG^{GlyT2} neurons of GlyT2::Cre male and female mice (Figure 5A, Supplementary Figure 4). We first demonstrate that PAG^{GlyT2} neurons do not modulate responses to mechanical stimuli (Figure 5B) and replicated our previous findings that PAG^{GlyT2} neurons bidirectionally modulate thermal nociception (Figure 5C-D). We then extended our initial analysis of open field behaviours and found bidirectional modulation of mobility (Figure 5E) and no difference in centre zone activity in males (Figure 5F), but changes in rearing and jumping behaviours. Time interval analysis reveals that in the first five minutes of the open field, rearing effects dominated while jumping was more strongly observed during the latter 15 minutes of the testing period (Figure 5G-J). Similar behaviours were observed in female mice (Supplementary Figure 5).

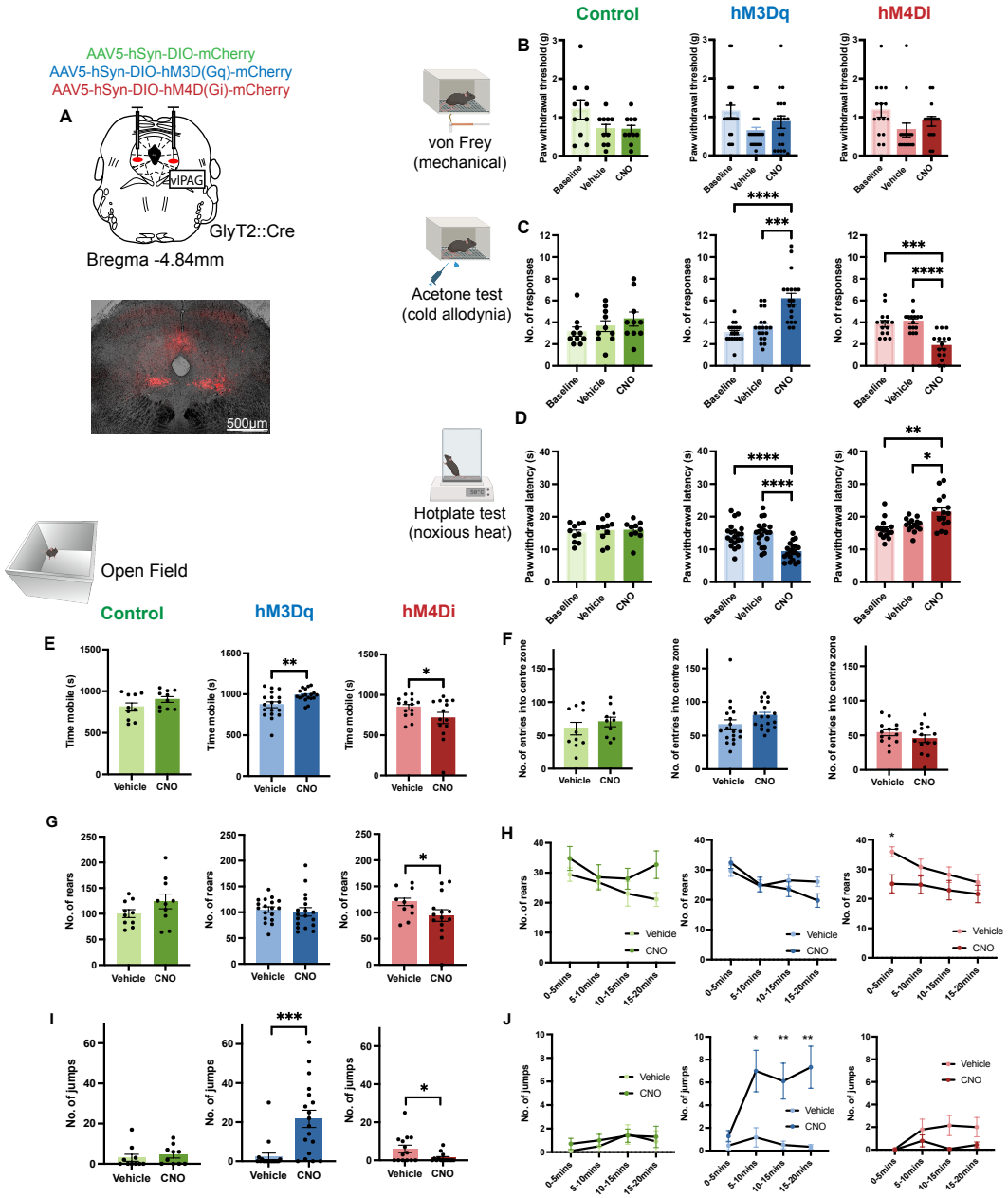


Figure 5: Chemogenetic modulation of PAG^{GlyT2} neurons bidirectionally modulates acute thermal nociception and locomotor activity in male mice. (A) Schematic and representative image showing bilateral stereotaxic injection and expression of the excitatory Cre-dependent DREADD into the vlPAG of GlyT2::Cre male mice, targeting PAG^{GlyT2} neurons. Unilaterally injected animals were also included in this experiment. (B) Chemogenetic modulation does not change paw withdrawal thresholds. (C) Chemogenetic activation (hM3D; blue) via CNO administration (3mg/kg; i.p.) resulted in increased responses in the acetone test, while inhibition (hM4D; red) via CNO administration (5mg/kg; i.p.) resulted in decreased responses. (D) On the hotplate test, chemogenetic activation (blue) caused a decrease in paw withdrawal latency (s) while inhibition (red) caused an increase. (E) Chemogenetic activation increased mobility (blue) while inhibition (red) reduced mobility in the open field test. (F) Number of entries into the

centre zone of the open field was unchanged following chemogenetic activation or inhibition of PAG^{GlyT2} neurons. (G) The number of rears was significantly reduced following inhibition of PAG^{GlyT2} neurons (red), and this was evident in the first five minutes of the open field test (H). (I-J) The number of jumps was significantly increased following chemogenetic activation (blue) while it decreased following chemogenetic inhibition (red) and this was mainly evident in the last fifteen minutes of the open field. Individual animals indicated (n= 10 (control), n=18 (excitatory), n=14 (inhibitory); mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To directly address if chemogenetic modulation of PAG^{GlyT2} neurons can modulate anxiety-like responses we used the light-dark test. This test relies on the natural aversion of mice to brightly illuminated areas and on their spontaneous exploration in novel environments (Riebe & Wotjak, 2012). In male mice, the entries and time spent in the light zone of the light-dark test was increased compared to control when PAG^{GlyT2} neurons were chemogenetically activated, whereas inhibition was associated with decreased time spent in the light zone (Figure 6A-B). In addition, the latency to first enter the dark zone was increased when PAG^{GlyT2} neurons were chemogenetically activated (Figure 6C). These findings indicate that PAG^{GlyT2} neurons bidirectionally modulate anxiety-like behaviors. To strengthen and validate this finding, we also assessed the ability of PAG^{GlyT2} activity to alter time spent in the open arms of the elevated plus maze in a subset of animals. Inhibition of PAG^{GlyT2} neurons significantly reduced entries and time spent in the open arms of the elevated plus maze while excitation of PAG^{GlyT2} neurons was associated with increased entries to explore an open illuminated space (open arms), although this did not reach significance (p=0.0653; Figure 6D-E). We carried out a parallel set of light-dark tests in female mice and similarly detected increased time spent in the light zone when PAG^{GlyT2} neurons were activated (Supplementary Figure 6). Taken together with the open field behaviours, we conclude that PAG^{GlyT2} neurons modulate anxiety-like behaviours, with activation and inhibition reducing and promoting anxiety-like behaviours, respectively.

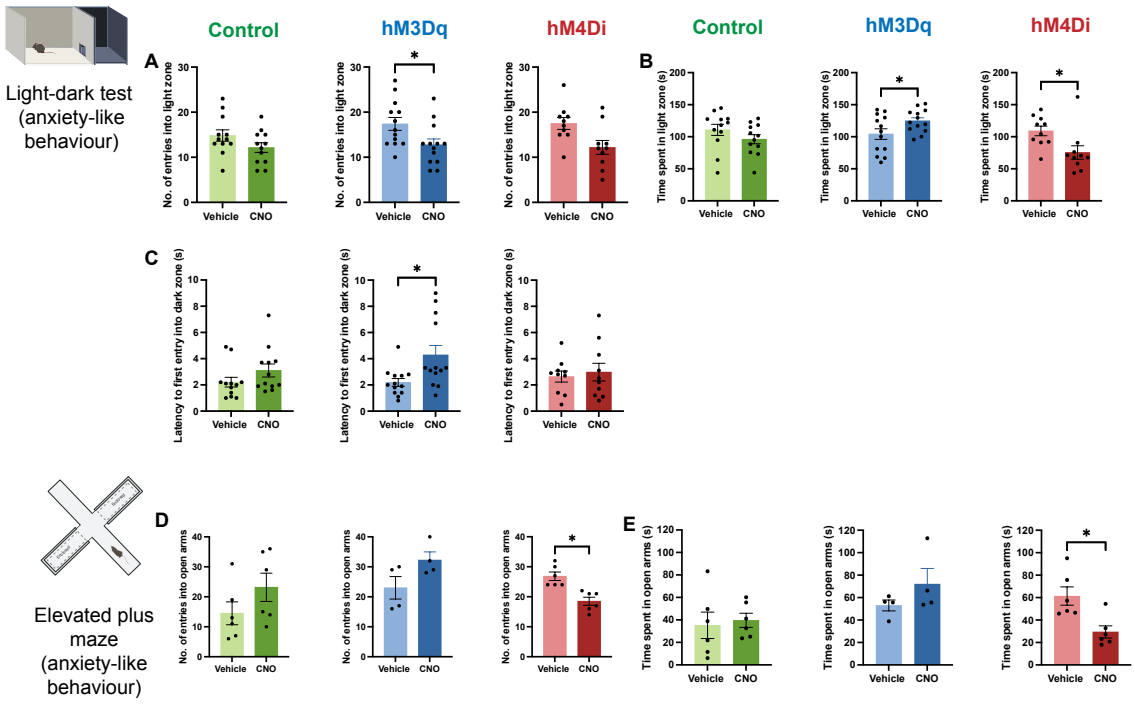


Figure 6: Chemogenetic modulation of PAG^{GlyT2} neurons induces anxiety-like behaviour in GlyT2::Cre male mice. (A) The number of entries in the light zone during the light dark test is significantly reduced following chemogenetic activation (hM3D, blue) of PAG^{GlyT2} neurons. (B) Time spent in the light zone is significantly increased (blue) and reduced (hM4D; red) following chemogenetic activation or inhibition, respectively. (C) The latency to first entry into the dark zone is significantly increased following chemogenetic activation (blue) of PAG^{GlyT2} cells. In the elevated plus maze, chemogenetic inhibition (red) caused a reduction in number of entries (D) and time spent (E) in the open arms. Individual animals indicated (n= 4- 13/group; mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Chemogenetic inhibition of PAG^{GlyT2} neurons is aversive

An increase in the willingness of an animal to spend time in the light zone is traditionally interpreted as an increased drive to explore novel environments. On the other hand, reduction of time spent in the light zone reflects increased anxiety-like behaviours, which should be seen as aversive. To test this assumption, we conducted a conditioned place aversion test in a mixed cohort of male and female GlyT2::Cre mice expressing control, hM3D or hM4D chemogenetic DREADDs (Figure 7A). In hM4Di injected mice, chemogenetic inhibition of PAG^{GlyT2} neurons was associated with a significant decrease in time spent in the CNO-paired zone on both post conditioning test days (2 and 8 days after conditioning). This decrease was also apparent when data was analysed as a difference score, supporting the validity of this finding (Figure 7B-G; Yates, 2023). No significant change was detected in the control or hM3Dq mice. These findings

are consistent with activation of PAG^{GlyT2} neurons promoting exploration (i.e. increased time in the light zone) and their inhibition causing an anxiety-like state (i.e. decreased time in the light zone and aversive).

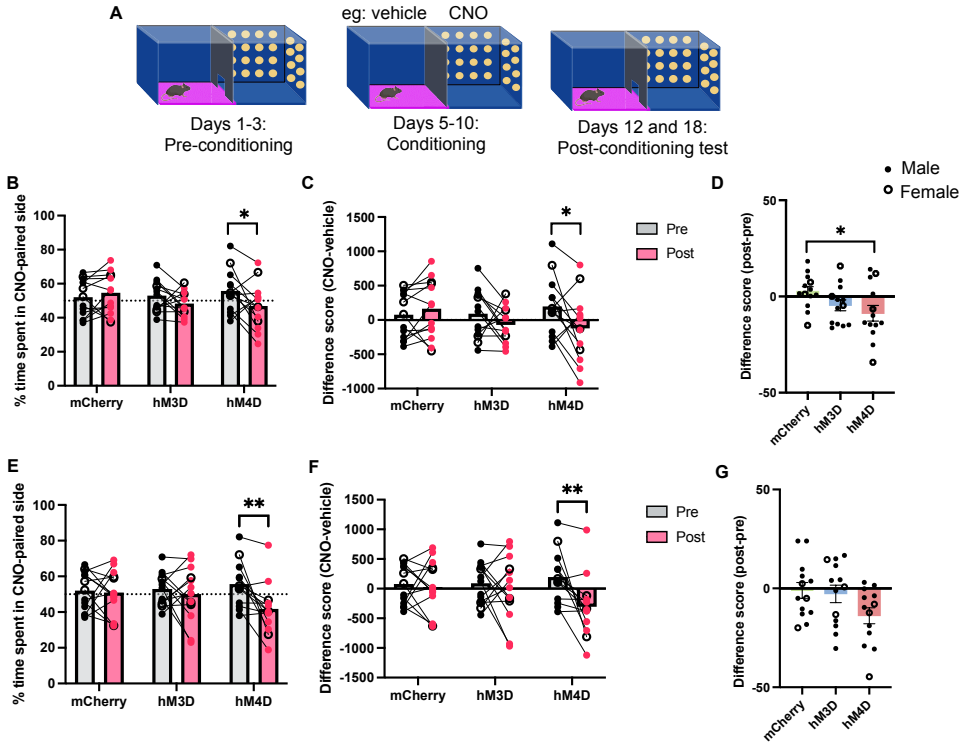


Figure 7: Conditioned place aversion shows that chemogenetic inhibition of PAG^{GlyT2} neurons is aversive in male and female GlyT2::Cre mice. (A) Schematic showing experimental timeline of conditioned place aversion test showing the animals had three days of pre-conditioning, followed by six days of conditioning and a post conditioning test on day 12 and 18 (all 30-minute duration). (B, E) Percentage of time spent in the CNO-paired side during the pre-conditioning (grey) and post conditioning (pink) of mCherry, hM3D and hM4D male (closed) and female (open) GlyT2::Cre mice, such that chemogenetic inhibition (hM4D) in the CNO-paired side reduced time spent on that side (Day 12 and 18). (C) Difference score (time spent in the CNO-paired side minus the time spent in the vehicle-paired side) between pre and post conditioning is significantly reduced following chemogenetic inhibition (hM4D) of PAG^{GlyT2} neurons (Day 12). (D) Percentage of time spent in the CNO-paired side between the post conditioning and pre-conditioning test is significantly reduced in the hM4D group compared to the mCherry group (Day 12). (E) On day 18, the percentage of time spent in the CNO-paired side during the pre-conditioning (grey) and post conditioning (pink) of mCherry, hM3D and hM4D male (closed point) and female (open point) GlyT2::Cre mice, such that chemogenetic inhibition (hM4D) in the CNO-paired side reduced time spent on that side. (F) Difference score (time spent in the CNO-paired side minus the time spent in the vehicle-paired side) between pre and post conditioning is unchanged following chemogenetic

modulation of PAG^{GlyT2} neurons (Day 18). (D) Percentage of time spent in the CNO-paired side between the post conditioning and pre-conditioning test is significantly reduced in the hM4D group compared to the mCherry group (Day 18). Individual animals indicated (n= 10 males and 3 females/group; mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5 Discussion

The PAG is a key integrator of sensory information that is central to shaping responses to pain and other stressors. The vlPAG is a key site for the analgesic actions of opioids, like morphine, such that inhibitory interneurons in the PAG disinhibit projection neurons in the descending in the descending pain modulatory pathway that can interrupt nociceptive signal in the spinal cord (Lau & Vaughan, 2014; Vaughan et al., 1997; Winters et al., 2022). However, current evidence suggests there are over 100 different neuronal subtypes within the PAG (Vaughn et al., 2022), and thus the role of the vlPAG has only begun to be appreciated (H. Zhang et al., 2024).

Here we provide the first comprehensive evidence that PAG^{GlyT2} neurons are a novel subpopulation of inhibitory PAG neurons. First, our electrophysiology and circuit tracing data indicates that unlike their interneuron counterparts, PAG^{GlyT2} neurons are inhibitory projection neurons that project to a range of brain regions not only in the pons and brainstem, but also in the hypothalamus and thalamus. This opens the possibility that they can directly modify neuronal function at a range of target regions that are known to be involved in emotional, motivational and autonomic responses to pain. Further, we demonstrate that PAG^{GlyT2} neurons can sense noxious and non-noxious aversive stimuli and show that in addition to thermal nociception, they modulate anxiety-like behaviours and aversion in males and females (Figure 8). Overall, this data suggests that PAG^{GlyT2} neurons are a functionally relevant neuronal subtype in the vlPAG that are involved in the coordination of complex behavioural responses and expands the computational potential of PAG function.

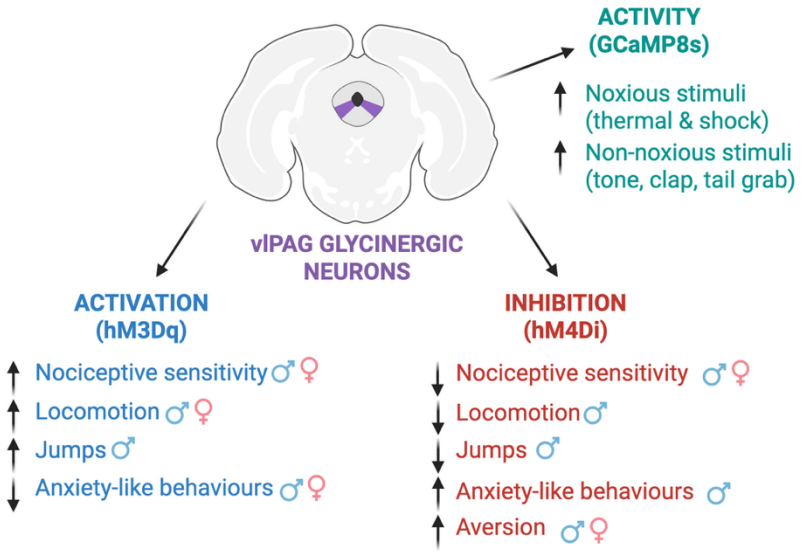


Figure 8: Summary of the role of PAG^{GlyT2} neurons. Created with BioRender.

PAG^{GlyT2} neurons are a subpopulation of GABAergic neurons that are likely to corelease GABA and glycine

Our finding that PAG^{GlyT2} neurons make up ~ 17% of inhibitory neurons in the vlPAG is consistent with previous studies which show they co-express established inhibitory neuronal markers (vGAT/VIAAT and GAD65/67) along with the precursor of endogenous opioids, proenkephalin (Kimmey et al., 2025; Lein et al., 2007; Tanaka & Ezure, 2004; Vaughn et al., 2022). As vGAT/VIAAT concentrates both GABA and glycine into synaptic vesicles, this expression profile suggests these neurons co-accumulate and co-release glycine with GABA and their dense core vesicle could release met-enkephalin (Aubrey et al., 2007; Jonas et al., 1998; Vaaga et al., 2014). The data presented is consistent with this possibility but met-enkephalin supply from PAG^{GlyT2} neurons has not been demonstrated and diversity in vesicular transmitter content or postsynaptic receptor expression is possible (Aubrey & Supplisson, 2018).

The role of PAG^{GlyT2} neurons is consistent with previously established roles of vlPAG inhibitory populations in both anxiety-like behaviours (Lowery-Gionta et al., 2018) and exploration (Reis et al., 2024). Considering this, our data suggests that chemogenetic activation of PAG^{GlyT2} neurons may not promote escape behaviours shown through jumping responses in the open field, as initially suggested in Assareh et al., 2023, but simply a locomotor response. This is consistent with the idea that is it was a defensive escape response this would likely be aversive,

of which activation is not. In addition, inhibitory neurons in the vlPAG have also been implicated in itch (Samineni et al., 2019), fear (Tovote et al., 2016), feeding (Hao et al., 2019), coughing (Chen et al., 2022) and sleep (Weber et al., 2018). This poses the question that composite collection of subpopulations of neurons in the vlPAG coordinate all these PAG mediated responses. Therefore, this study gives evidence that future experiments should consider the interplay of different neuronal populations and complex behaviours and not interpret their findings in isolation.

PAG^{GlyT2} neurons are inhibitory projection neurons

We also showed that PAG^{GlyT2} neurons are mostly projection neurons that have extensive axonal projections to a range of descending and ascending regions that are known to be involved with a range of complex behaviours. We hypothesise that PAG^{GlyT2} neurons modulate nociception via the descending pathway however the classical understanding of PAG circuitry has been challenged previously. Indeed, inhibitory projections out of the PAG has been shown (Rampon et al., 1999; Reis et al., 2024; Varga et al., 2019) and the classic framework of the descending analgesic pathway has been extended (Winters et al., 2022). Recent discoveries into new glycine receptors also add to the increased potential of these projections (Fenech et al., 2024; Grand et al., 2018; Laboute et al., 2023; Otsu et al., 2019). Therefore, the process by which PAG^{GlyT2} neurons modulate nociception as well as affective behaviours may be more complex, adding to the potential of PAG mediated circuits.

Limitations

There is increasing evidence that there are substantial sex differences in pain responses and pathways in animals (Mogil, 2009) and humans (Greenspan et al., 2007), including in the PAG (Jiang et al., 2022; Linnman et al., 2012; Loyd et al., 2007; Tonsfeldt et al., 2016; Yu et al., 2021). As such, we extended our initial preliminary experiment in females (Assareh et al., 2023) to robustly examine nociception and anxiety-like behaviours in both males and females. Our data, although not exactly mirrored between sexes, suggests that PAG^{GlyT2} neurons do not contribute to sex differences in the PAG. We did find subtle differences in behaviours between the male and female datasets; however, this could be explained by innate differences in ethological behaviours (Meseguer Henarejos et al., 2020; Sensini et al., 2020). Female rodents

are historically ignored in preclinical research due to concerns about increased variability in female mice, however this has been debunked (Kaluve et al., 2022). This study does not provide strong evidence of sex differences in PAG^{GlyT2} neurons, but future studies should use both sexes to ensure that if differences arise, they can be discovered.

Overall, PAG^{GlyT2} neurons reveal exciting insights into the potential of a small subpopulation of neurons that could contribute widely to the overall pain experience. This study expands the potential of glycinergic neurotransmission in the brain as well as midbrain circuitry as a whole. Thus, revealing how the brain can compute complex behaviours.

3.6 References (Chapter 3)

Anderson, W. B., Gould, M. J., Torres, R. D., Mitchell, V. A., & Vaughan, C. W. (2014). Actions of the dual FAAH/MAGL inhibitor JZL195 in a murine inflammatory pain model. *Neuropharmacology*, *81*, 224–230. https://doi.org/10.1016/j.neuropharm.2013.12.018

Arico, C., Bagley, E. E., Carrive, P., Assareh, N., & McNally, G. P. (2017). Effects of chemogenetic excitation or inhibition of the ventrolateral periaqueductal gray on the acquisition and extinction of Pavlovian fear conditioning. *Neurobiology of Learning and Memory*, *144*, 186–197. https://doi.org/10.1016/j.nlm.2017.07.006

Assareh, N., Fenech, C., Power, R., Uddin, M. N., Otsu, Y., & Aubrey, K. R. (2023). Bidirectional Modulation of Nociception by GlyT2+ Neurons in the Ventrolateral Periaqueductal Gray. *eNeuro*, *10*(6). https://doi.org/10.1523/ENEURO.0069-23.2023

Assareh, N., Sokolaj, E. E., Sadia, S., Anderson, K. E., Frith, C., Walls, O. B., Mitchell, V. A., Vaughan, C. W., & Winters, B. L. (2025). Fear-learning is altered in a mouse neuropathic pain model. *Frontiers in Pain Research*, *6*. https://doi.org/10.3389/fpain.2025.1648374

Aubrey, K. R., Rossi, F. M., Ruivo, R., Alboni, S., Bellenchi, G. C., Le Goff, A., Gasnier, B., & Supplisson, S. (2007). The transporters GlyT2 and VIAAT cooperate to determine the vesicular glycinergic phenotype. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *27*(23), 6273–6281. https://doi.org/10.1523/JNEUROSCI.1024-07.2007

Aubrey, K. R., & Supplisson, S. (2018). Heterogeneous Signaling at GABA and Glycine Co-releasing Terminals. *Frontiers in Synaptic Neuroscience*, *10*. https://doi.org/10.3389/fnsyn.2018.00040

Bailey, K. R., & Crawley, J. N. (2009). Anxiety-Related Behaviors in Mice. In J. J. Buccafusco (Ed.), *Methods of Behavior Analysis in Neuroscience* (2nd ed.). CRC Press/Taylor & Francis. http://www.ncbi.nlm.nih.gov/books/NBK5221/

Bonin, R. P., Bories, C., & De Koninck, Y. (2014). A Simplified Up-Down Method (SUDO) for Measuring Mechanical Nociception in Rodents Using von Frey Filaments. *Molecular Pain*, *10*, 1744-8069-10–26. https://doi.org/10.1186/1744-8069-10-26

Bourin, M., & Hascoët, M. (2003). The mouse light/dark box test. *European Journal of Pharmacology*, *463*(1–3), 55–65. https://doi.org/10.1016/s0014-2999(03)01274-3

Cameron, A. A., Khan, I. A., Westlund, K. N., Cliffer, K. D., & Willis, W. D. (1995). The efferent projections of the periaqueductal gray in the rat: A Phaseolus vulgaris-leucoagglutinin study. I. Ascending projections. *The Journal of Comparative Neurology*, *351*(4), 568–584. https://doi.org/10.1002/cne.903510407

Cameron, A. A., Khan, I. A., Westlund, K. N., & Willis, W. D. (1995). The efferent projections of the periaqueductal gray in the rat: A Phaseolus vulgaris-leucoagglutinin study. II. Descending projections. *Journal of Comparative Neurology*, *351*(4), 585–601. https://doi.org/10.1002/cne.903510408

Chen, Z., Lin, M.-T., Zhan, C., Zhong, N.-S., Mu, D., Lai, K.-F., & Liu, M. J. (2022). A descending pathway emanating from the periaqueductal gray mediates the development of cough-like hypersensitivity. *iScience*, *25*(1). https://doi.org/10.1016/j.isci.2021.103641

Chieng, B., & Christie, M. J. (1994). Hyperpolarization by opioids acting on mu-receptors of a sub-population of rat periaqueductal gray neurones in vitro. *British Journal of Pharmacology*, *113*(1), 121–128. https://doi.org/10.1111/j.1476-5381.1994.tb16183.x

Deng, H., Wu, Y., Gao, P., Kong, D., Pan, C., Xu, S., Tang, D., Jiao, Y., Wen, D., & Yu, W. (2023). Preoperative Pain Facilitates Postoperative Cognitive Dysfunction via Periaqueductal Gray Matter–Dorsal Raphe Circuit. *Neuroscience*, *524*, 209–219. https://doi.org/10.1016/j.neuroscience.2023.03.019

Espejo, E. F., Stinus, L., Cador, M., & Mir, D. (1994). Effects of morphine and naloxone on behaviour in the hot plate test: An ethopharmacological study in the rat. *Psychopharmacology*, *113*(3), 500–510. https://doi.org/10.1007/BF02245230

Fenech, C., Winters, B. L., Otsu, Y., & Aubrey, K. R. (2024). Supraspinal glycinergic neurotransmission in pain: A scoping review of current literature. *Journal of Neurochemistry*, *168*(11), 3663–3684. https://doi.org/10.1111/jnc.16191

Foster, E., Wildner, H., Tudeau, L., Haueter, S., Ralvenius, W. T., Jegen, M., Johannssen, H., Hösli, L., Haenraets, K., Ghanem, A., Conzelmann, K.-K., Bösl, M., & Zeilhofer, H. U. (2015). Targeted Ablation, Silencing, and Activation Establish Glycinergic Dorsal Horn Neurons as Key Components of a Spinal Gate for Pain and Itch. *Neuron*, *85*(6), 1289–1304. https://doi.org/10.1016/j.neuron.2015.02.028

François, A., Low, S. A., Sypek, E. I., Christensen, A. J., Sotoudeh, C., Beier, K. T., Ramakrishnan, C., Ritola, K. D., Sharif-Naeini, R., Deisseroth, K., Delp, S. L., Malenka, R. C., Luo, L., Hantman, A. W., & Scherrer, G. (2017). A Brainstem-Spinal Cord Inhibitory Circuit for Mechanical Pain Modulation by GABA and Enkephalins. *Neuron*, *93*(4), 822-839.e6. https://doi.org/10.1016/j.neuron.2017.01.008

Grand, T., Abi Gerges, S., David, M., Diana, M. A., & Paoletti, P. (2018). Unmasking GluN1/GluN3A excitatory glycine NMDA receptors. *Nature Communications*, *9*(1), 4769. https://doi.org/10.1038/s41467-018-07236-4

Greenspan, J. D., Craft, R. M., LeResche, L., Arendt-Nielsen, L., Berkley, K. J., Fillingim, R. B., Gold, M. S., Holdcroft, A., Lautenbacher, S., Mayer, E. A., Mogil, J. S., Murphy, A. Z., Traub, R. J., & the Consensus Working Group of the Sex, G. (2007). Studying sex and gender differences in pain and analgesia: A consensus report. *PAIN*, *132*, S26. https://doi.org/10.1016/j.pain.2007.10.014

Hao, S., Yang, H., Wang, X., He, Y., Xu, H., Wu, X., Pan, L., Liu, Y., Lou, H., Xu, H., Ma, H., Xi, W., Zhou, Y., Duan, S., & Wang, H. (2019). The Lateral Hypothalamic and BNST GABAergic Projections to the Anterior Ventrolateral Periaqueductal Gray Regulate Feeding. *Cell Reports*, *28*(3), 616-624.e5. https://doi.org/10.1016/j.celrep.2019.06.051

Ho, Y.-C., Lin, T.-B., Hsieh, M.-C., Lai, C.-Y., Chou, D., Chau, Y.-P., Chen, G.-D., & Peng, H.-Y. (2018). Periaqueductal Gray Glutamatergic Transmission Governs Chronic Stress-Induced Depression. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *43*(2), 302–312. https://doi.org/10.1038/npp.2017.199

Hurst, J. L., & West, R. S. (2010). Taming anxiety in laboratory mice. *Nature Methods*, *7*(10), 825–826. https://doi.org/10.1038/nmeth.1500

Jiang, Z., Wang, Q., Zhao, J., Wang, J., Li, Y., Dai, W., Zhang, X., Fang, Z., Hou, W., & Xiong, L. (2022). Sex-specific cannabinoid 1 receptors on GABAergic neurons in the ventrolateral periaqueductal gray mediate analgesia in mice. *Journal of Comparative Neurology*, *530*(13), 2315–2334. https://doi.org/10.1002/cne.25334

Jonas, P., Bischofberger, J., & Sandkühler, J. (1998). Corelease of two fast neurotransmitters at a central synapse. *Science (New York, N.Y.)*, *281*(5375), 419–424. https://doi.org/10.1126/science.281.5375.419

Kaluve, A. M., Le, J. T., & Graham, B. M. (2022). Female rodents are not more variable than male rodents: A meta-analysis of preclinical studies of fear and anxiety. *Neuroscience & Biobehavioral Reviews*, *143*, 104962. https://doi.org/10.1016/j.neubiorev.2022.104962

Keay, K. A., & Bandler, R. (2015). Chapter 10—Periaqueductal Gray. In G. Paxinos (Ed.), *The Rat Nervous System (Fourth Edition)* (pp. 207–221). Academic Press. https://doi.org/10.1016/B978-0-12-374245-2.00010-3

Kimmey, B. A., Ejoh, L., Shangloo, L., Wojick, J. A., Chehimi, S. N., McCall, N. M., Oswell, C. S., Mahmood, M., Yang, L., Samineni, V. K., Ramakrishnan, C., Deisseroth, K., Crist, R. C., Reiner, B. C., Tian, L., & Corder, G. (2025). Convergent state-control of endogenous opioid analgesia. *bioRxiv*, 2025.01.03.631111. https://doi.org/10.1101/2025.01.03.631111

Laboute, T., Zucca, S., Holcomb, M., Patil, D. N., Garza, C., Wheatley, B. A., Roy, R. N., Forli, S., & Martemyanov, K. A. (2023). Orphan receptor GPR158 serves as a metabotropic glycine receptor: mGlyR. *Science*, *379*(6639), 1352–1358. https://doi.org/10.1126/science.add7150

Lau, B. K., & Vaughan, C. W. (2014). Descending modulation of pain: The GABA disinhibition hypothesis of analgesia. *Current Opinion in Neurobiology*, *29*, 159–164. https://doi.org/10.1016/j.conb.2014.07.010

Lau, B. K., Winters, B. L., & Vaughan, C. W. (2020). Opioid presynaptic disinhibition of the midbrain periaqueductal grey descending analgesic pathway. *British Journal of Pharmacology*, *177*(10), 2320–2332. https://doi.org/10.1111/bph.14982

Lee, M. T., Peng, W.-H., Wu, C.-C., Kan, H.-W., Wang, D.-W., Teng, Y.-N., & Ho, Y.-C. (2023). Impaired Ventrolateral Periaqueductal Gray-Ventral Tegmental area Pathway Contributes to Chronic Pain-Induced Depression-Like Behavior in Mice. *Molecular Neurobiology*, *60*(10), 5708–5724. https://doi.org/10.1007/s12035-023-03439-z

Lein, E. S., Hawrylycz, M. J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A. F., Boguski, M. S., Brockway, K. S., Byrnes, E. J., Chen, L., Chen, L., Chen, T.-M., Chin, M. C., Chong, J., Crook, B. E., Czaplinska, A., Dang, C. N., Datta, S., … Jones, A. R. (2007). Genome-wide atlas of gene expression in the adult mouse brain. *Nature*, *445*(7124), 168–176. https://doi.org/10.1038/nature05453

Linnman, C., Beucke, J.-C., Jensen, K. B., Gollub, R. L., & Kong, J. (2012). Sex similarities and differences in pain-related periaqueductal gray connectivity. *Pain*, *153*(2), 444–454. https://doi.org/10.1016/j.pain.2011.11.006

Lowery-Gionta, E. G., DiBerto, J., Mazzone, C. M., & Kash, T. L. (2018). GABA neurons of the ventral periaqueductal gray area modulate behaviors associated with anxiety and conditioned fear. *Brain Structure and Function*, *223*(8), 3787–3799. https://doi.org/10.1007/s00429-018-1724-z

Loyd, D. R., Morgan, M. M., & Murphy, A. Z. (2007). Morphine preferentially activates the periaqueductal gray–rostral ventromedial medullary pathway in the male rat: A potential mechanism for sex differences in antinociception. *Neuroscience*, *147*(2), 456–468. https://doi.org/10.1016/j.neuroscience.2007.03.053

Meseguer Henarejos, A. B., Popović, N., Bokonjić, D., Morales-Delgado, N., Alonso, A., Caballero Bleda, M., & Popović, M. (2020). Sex and Time-of-Day Impact on Anxiety and Passive Avoidance Memory Strategies in Mice. *Frontiers in Behavioral Neuroscience*, *14*. https://doi.org/10.3389/fnbeh.2020.00068

Mitchell, V. A., Harley, J., Casey, S. L., Vaughan, A. C., Winters, B. L., & Vaughan, C. W. (2021). Oral efficacy of Δ(9)-tetrahydrocannabinol and cannabidiol in a mouse neuropathic pain model. *Neuropharmacology*, *189*, 108529. https://doi.org/10.1016/j.neuropharm.2021.108529

Mogil, J. S. (2009). Animal models of pain: Progress and challenges. *Nature Reviews Neuroscience*, *10*(4), 283–294. https://doi.org/10.1038/nrn2606

Osborne, P. B., Vaughan, C. W., Wilson, H. I., & Christie, M. J. (1996). Opioid inhibition of rat periaqueductal grey neurones with identified projections to rostral ventromedial medulla in vitro. *The Journal of Physiology*, *490*(2), 383–389. https://doi.org/10.1113/jphysiol.1996.sp021152

Otsu, Y., Darcq, E., Pietrajtis, K., Matyas, F., Schwartz, E., Bessaih, T., Abi Gerges, S., Rousseau, C. V., Grand, T., Dieudonné, S., Paoletti, P., Acsady, L., Agulhon, C., Kieffer, B. L., & Diana, M. A. (2019). Control of aversion by glycine-gated GluN1/GluN3A NMDA receptors in the adult Medial Habenula. *Science (New York, N.Y.)*, *366*(6462), 250–254. https://doi.org/10.1126/science.aax1522

Philipsberg, P. A., Christenson Wick, Z., Diego, K. S., Vaughan, N., Galas, A., Jurkowski, A., Feng, Y., Vetere, L. M., Chen, L., Soler, I., Cai, D. J., & Shuman, T. (2023). Chronotate: An open-source tool for manual timestamping and quantification of animal behavior. *Neuroscience Letters*, *814*, 137461. https://doi.org/10.1016/j.neulet.2023.137461

Rampon, C., Luppi, P. H., Fort, P., Peyron, C., & Jouvet, M. (1996). Distribution of glycine-immunoreactive cell bodies and fibers in the rat brain. *Neuroscience*, *75*(3), 737–755. https://doi.org/10.1016/0306-4522(96)00278-3

Rampon, C., Peyron, C., Gervasoni, D., Pow, D. V., Luppi, P.-H., & Fort, P. (1999). Origins of the glycinergic inputs to the rat locus coeruleus and dorsal raphe nuclei: A study combining retrograde tracing with glycine immunohistochemistry. *European Journal of Neuroscience*, *11*(3), 1058–1066. https://doi.org/10.1046/j.1460-9568.1999.00511.x

Reis, F. M. C. V., Maesta-Pereira, S., Ollivier, M., Schuette, P. J., Sethi, E., Miranda, B. A., Iniguez, E., Chakerian, M., Vaughn, E., Sehgal, M., Nguyen, D. C. T., Yuan, F. T. H., Torossian, A., Ikebara, J. M., Kihara, A. H., Silva, A. J., Kao, J. C., Khakh, B. S., & Adhikari, A. (2024). Control of feeding by a bottom-up midbrain-subthalamic pathway. *Nature Communications*, *15*(1), 2111. https://doi.org/10.1038/s41467-024-46430-5

Riebe, C. J., & Wotjak, C. T. (2012). A Practical Guide to Evaluating Anxiety-Related Behavior in Rodents. In A. Szallasi & T. Bíró (Eds.), *TRP Channels in Drug Discovery: Volume II* (pp. 167–185). Humana Press. https://doi.org/10.1007/978-1-62703-095-3_10

Samineni, V. K., Grajales-Reyes, J. G., Sundaram, S. S., Yoo, J. J., & Gereau, R. W. (2019). Cell type-specific modulation of sensory and affective components of itch in the periaqueductal gray. *Nature Communications*, *10*(1), 4356. https://doi.org/10.1038/s41467-019-12316-0

Scicluna, R. L., Everett, N. A., Badolato, C. J., Wilson, B. B., & Bowen, M. T. (2024). Effects of CB1 receptor negative allosteric modulator Org27569 on oxycodone withdrawal symptoms in mice. *Psychopharmacology*, *241*(8), 1705–1717. https://doi.org/10.1007/s00213-024-06591-z

Sensini, F., Inta, D., Palme, R., Brandwein, C., Pfeiffer, N., Riva, M. A., Gass, P., & Mallien, A. S. (2020). The impact of handling technique and handling frequency on laboratory mouse welfare is sex-specific. *Scientific Reports*, *10*(1), 17281. https://doi.org/10.1038/s41598-020-74279-3

Tanaka, I., & Ezure, K. (2004). Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. *Neuroscience Research*, *49*(2), 165–178. https://doi.org/10.1016/j.neures.2004.02.007

Tonsfeldt, K. J., Suchland, K. L., Beeson, K. A., Lowe, J. D., Li, M., & Ingram, S. L. (2016). Sex Differences in GABAA Signaling in the Periaqueductal Gray Induced by Persistent Inflammation. *Journal of Neuroscience*, *36*(5), 1669–1681. https://doi.org/10.1523/JNEUROSCI.1928-15.2016

Tovote, P., Esposito, M. S., Botta, P., Chaudun, F., Fadok, J. P., Markovic, M., Wolff, S. B. E., Ramakrishnan, C., Fenno, L., Deisseroth, K., Herry, C., Arber, S., & Lüthi, A. (2016). Midbrain circuits for defensive behaviour. *Nature*, *534*(7606), 206–212. https://doi.org/10.1038/nature17996

Vaaga, C. E., Borisovska, M., & Westbrook, G. L. (2014). Dual-transmitter neurons: Functional implications of co-release and co-transmission. *Current Opinion in Neurobiology*, *0*, 25–32. https://doi.org/10.1016/j.conb.2014.04.010

Varga, E., Farkas, E., Zséli, G., Kádár, A., Venczel, A., Kővári, D., Németh, D., Máté, Z., Erdélyi, F., Horváth, A., Szenci, O., Watanabe, M., Lechan, R. M., Gereben, B., & Fekete, C. (2019). Thyrotropin-Releasing-Hormone-Synthesizing Neurons of the Hypothalamic Paraventricular Nucleus Are Inhibited by Glycinergic Inputs. *Thyroid: Official Journal of the American Thyroid Association*, *29*(12), 1858–1868. https://doi.org/10.1089/thy.2019.0357

Vaughan, C. W., Ingram, S. L., Connor, M. A., & Christie, M. J. (1997). How opioids inhibit GABA-mediated neurotransmission. *Nature*, *390*(6660), 611–614. https://doi.org/10.1038/37610

Vaughn, E., Eichhorn, S., Jung, W., Zhuang, X., & Dulac, C. (2022). *Three-dimensional Interrogation of Cell Types and Instinctive Behavior in the Periaqueductal Gray* (p. 2022.06.27.497769). bioRxiv. https://doi.org/10.1101/2022.06.27.497769

Weber, F., Hoang Do, J. P., Chung, S., Beier, K. T., Bikov, M., Saffari Doost, M., & Dan, Y. (2018). Regulation of REM and Non-REM Sleep by Periaqueductal GABAergic Neurons. *Nature Communications*, *9*(1), 354. https://doi.org/10.1038/s41467-017-02765-w

Winters, B. L., Lau, B. K., & Vaughan, C. W. (2022). Cannabinoids and Opioids Differentially Target Extrinsic and Intrinsic GABAergic Inputs onto the Periaqueductal Grey Descending Pathway. *Journal of Neuroscience*, *42*(41), 7744–7756. https://doi.org/10.1523/JNEUROSCI.0997-22.2022

Yang, L., Lu, J., Guo, J., Chen, J., Xiong, F., Wang, X., Chen, L., & Yu, C. (2022). Ventrolateral Periaqueductal Gray Astrocytes Regulate Nociceptive Sensation and Emotional Motivation in Diabetic Neuropathic Pain. *Journal of Neuroscience*, *42*(43), 8184–8199. https://doi.org/10.1523/JNEUROSCI.0920-22.2022

Yates, J. R. (2023). Quantifying conditioned place preference: A review of current analyses and a proposal for a novel approach. *Frontiers in Behavioral Neuroscience*, *17*. https://doi.org/10.3389/fnbeh.2023.1256764

Yoon, C., Wook, Y. Y., Sik, N. H., Ho, K. S., & Mo, C. J. (1994). Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. *PAIN*, *59*(3), 369. https://doi.org/10.1016/0304-3959(94)90023-X

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

Zafra, F., Ibáñez, I., & Giménez, C. (2016). Glycinergic transmission: Glycine transporter GlyT2 in neuronal pathologies. *Neuronal Signaling*, *1*(1), NS20160009. https://doi.org/10.1042/NS20160009

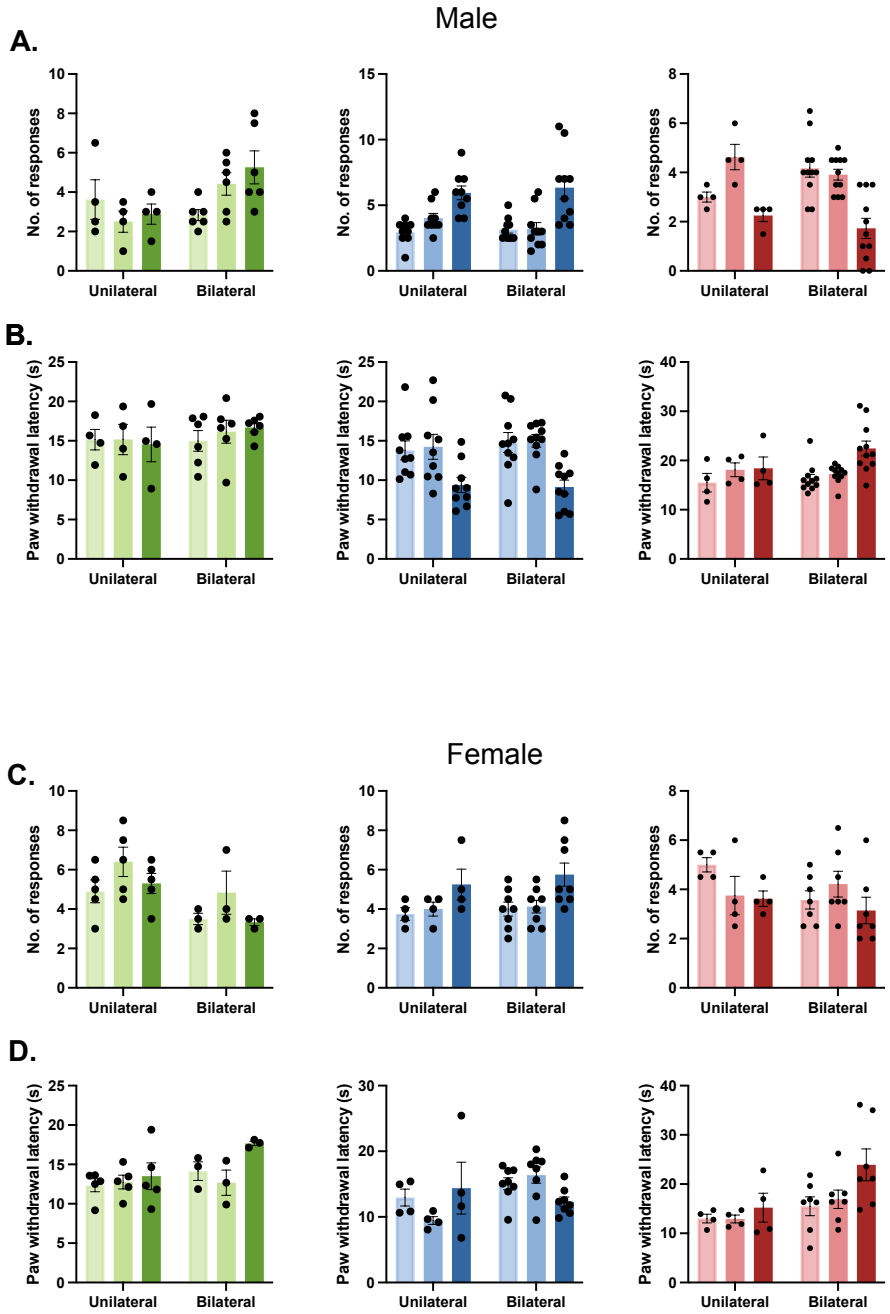
Zeilhofer, H. U. (2005). The glycinergic control of spinal pain processing. *Cellular and Molecular Life Sciences: CMLS*, *62*(18), 2027–2035. https://doi.org/10.1007/s00018-005-5107-2

Zeilhofer, H. U., Studler, B., Arabadzisz, D., Schweizer, C., Ahmadi, S., Layh, B., Bösl, M. R., & Fritschy, J.-M. (2005). Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. *The Journal of Comparative Neurology*, *482*(2), 123–141. https://doi.org/10.1002/cne.20349

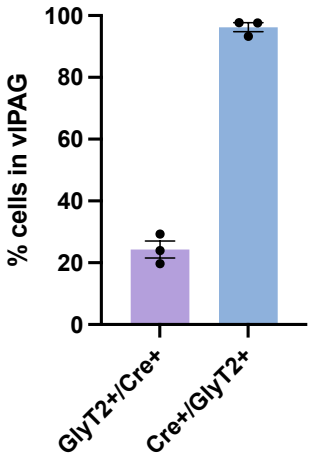
Zhang, H., Zhu, Z., Ma, W.-X., Kong, L.-X., Yuan, P.-C., Bu, L.-F., Han, J., Huang, Z.-L., & Wang, Y.-Q. (2024). The contribution of periaqueductal gray in the regulation of physiological and pathological behaviors. *Frontiers in Neuroscience*, *18*, 1380171. https://doi.org/10.3389/fnins.2024.1380171

Zhang, Y., Rózsa, M., Liang, Y., Bushey, D., Wei, Z., Zheng, J., Reep, D., Broussard, G. J., Tsang, A., Tsegaye, G., Narayan, S., Obara, C. J., Lim, J.-X., Patel, R., Zhang, R., Ahrens, M. B., Turner, G. C., Wang, S. S.-H., Korff, W. L., … Looger, L. L. (2023). Fast and sensitive GCaMP calcium indicators for imaging neural populations. *Nature*, *615*(7954), 884–891. https://doi.org/10.1038/s41586-023-05828-9

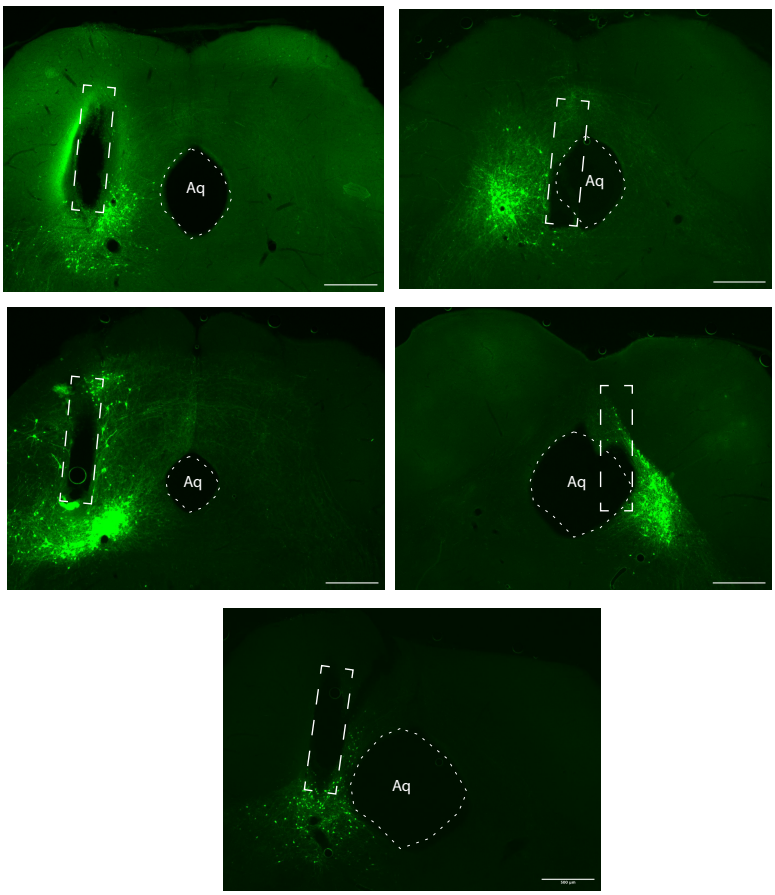
3.7 Supplementary Data



Supplementary Figure 1: Chemogenetic activation or inhibition with unilaterally or bilaterally injected GlyT2::Cre female and male mice. For the acetone test (A) and hotplate test (B), there is no significant difference between CNO administered male mice between unilateral and bilateral injected animals for control (mCherry; green), excitatory (hM3D; blue) and inhibitory (hM4D; red). For female CNO administered mice, there is no significant difference between unilateral and bilateral injected animals for all three groups regarding the acetone (C) and hotplate test (D). Individual animals indicated (n= 3- 11/group; mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

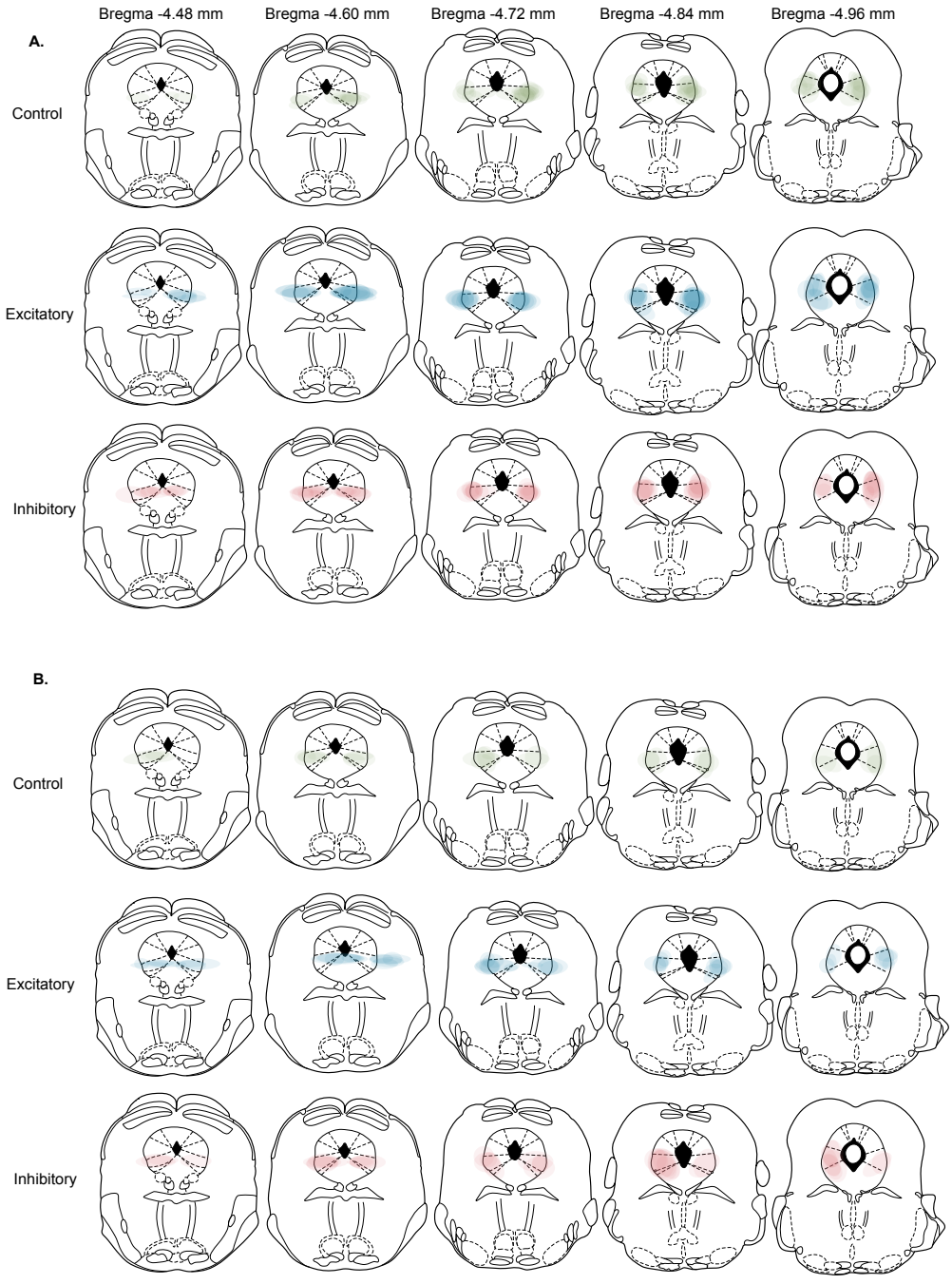


Supplementary Figure 2: FISH labelling of Cre+ neurons in GlyT2::Cre mice. Quantification of percentage of GlyT2 positive cells that were co-labelled with Cre (blue) and percentage of Cre positive cells that were co-labelled with GlyT2 following fluorescent *in situ* hybridisation (FISH) of GlyT2::Cre mice (n=3 animals; mean +/ SEM). vlPAG: ventrolateral periaqueductal grey.

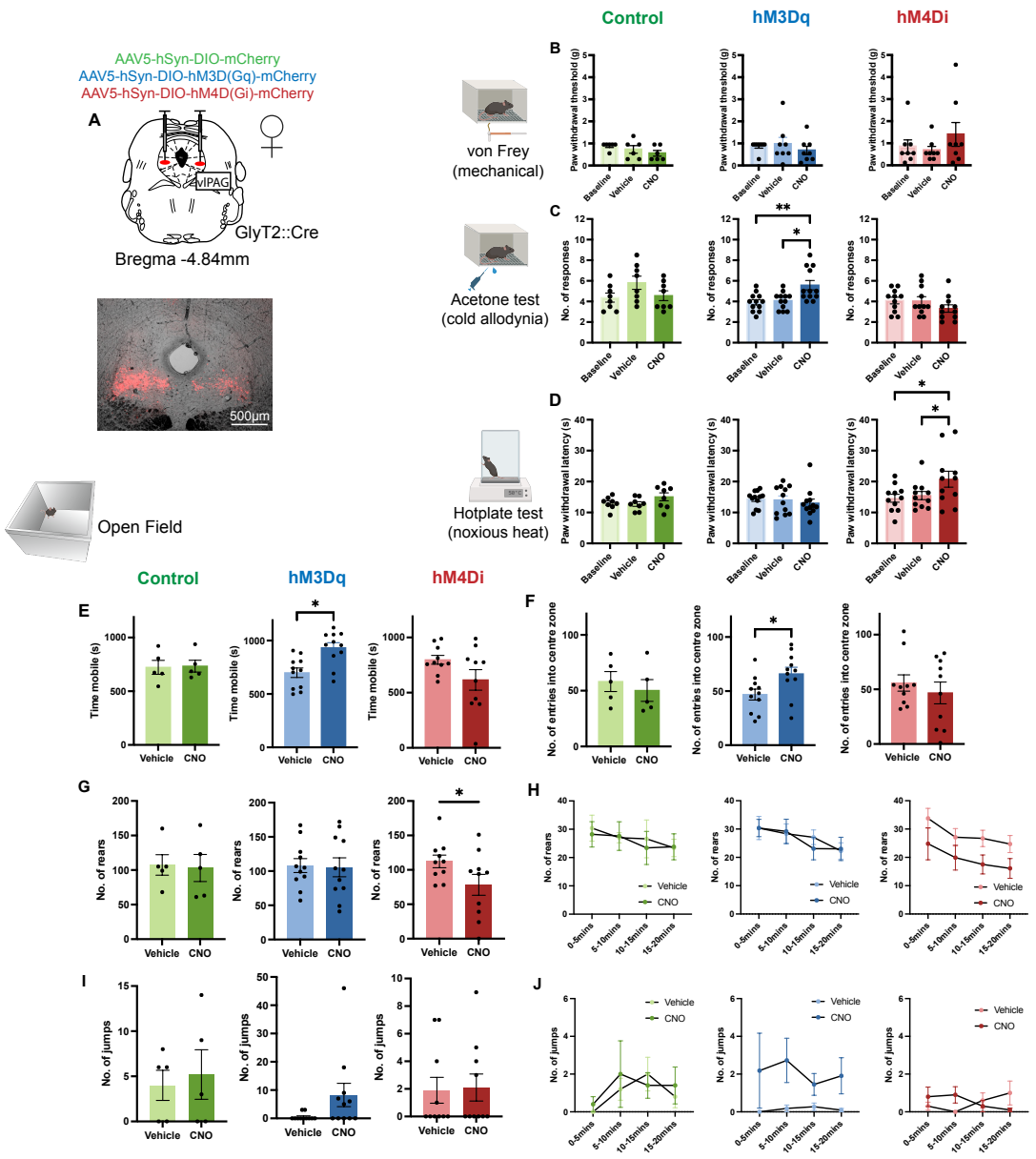


Supplementary Figure 3: Fibre and injection placement for *in vivo* fibre photometry experiment. Representative microscope image showing middle of injection site and fibre optic cannula

placement for fibre photometry experiment. n=1 animal unable to obtain image. Scale bar= 500µm. Magnification: 2.5X. Aq: Cerebral aqueduct.

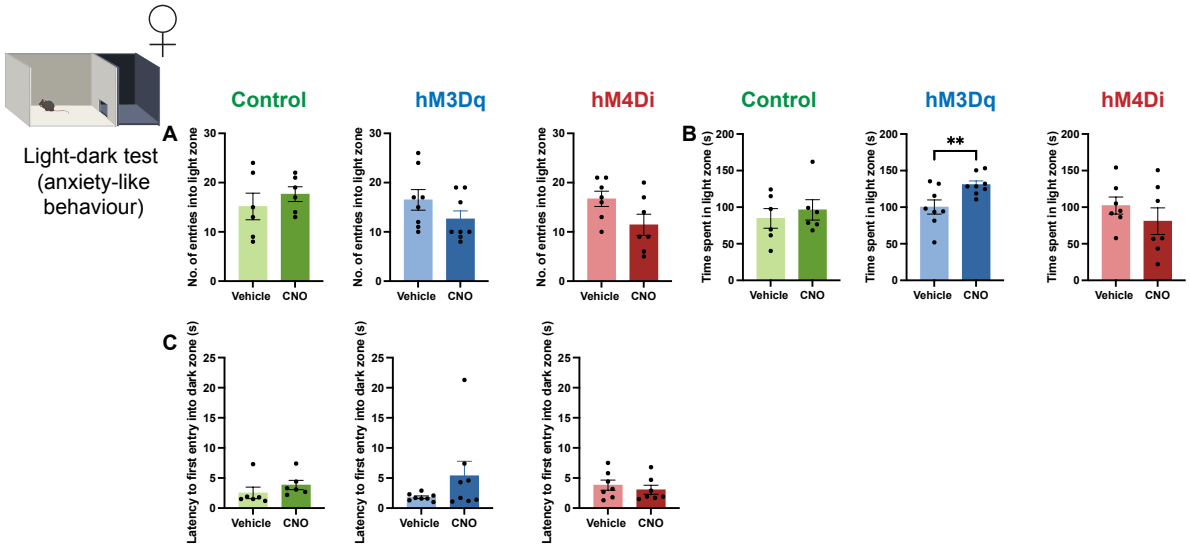


Supplementary Figure 4: Viral vector expression for all chemogenetic experiments. (A) Schematic showing expression of PAG^{GlyT2} neurons across all male and female (B) mice for control (mCherry; green), excitatory (hM3Dq; blue) and inhibitory (hM4Di; red) groups, with 10% opacity for expression spread per bregma section for each animal.



Supplementary Figure 5: Chemogenetic modulation of PAG^{GlyT2} neurons bidirectionally modulates acute thermal nociception and locomotor activity in female mice. (A) Schematic and representative image showing bilateral stereotaxic injection of the excitatory Cre-dependent DREADD into the vlPAG of GlyT2::Cre female mice, targeting PAG^{GlyT2} neurons. Unilaterally injected animals were also included in this experiment. (B) Chemogenetic modulation does not change paw withdrawal thresholds (C) Chemogenetic activation (hM3D; blue) via CNO administration (3mg/kg; i.p.) resulted in increased responses in the acetone test. (D) On the hotplate test, chemogenetic inhibition (hM4D; red) caused an increase in paw withdrawal latency. (E) Chemogenetic activation increased mobility (blue) in the open field test. (F) Number of entries into the centre zone of the open field was significantly increased following chemogenetic activation of PAG^{GlyT2} cells. (G) The number of rears was significantly reduced following inhibition of PAG^{GlyT2} neurons (red), and this effect was not specific to a certain time period during the open field test (H). (I, J) The number of jumps was unchanged following chemogenetic modulation in the open

field test. Individual animals indicated (n= 6-8 (control), n=8-12 (excitatory), n=8-11 (inhibitory); mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure 6: Chemogenetic activation of PAG^{GlyT2} neurons decreases anxiety-like behaviour in GlyT2::Cre female mice. (A) Chemogenetic modulation of PAG^{GlyT2} neurons does not change number of entries into the light zone during the light dark test. (B) Chemogenetic activation (hM3D; blue) significantly increases the time spent in the light zone in CNO injected female mice when compared to vehicle. (C) The latency to first entry into the dark zone is unchanged following chemogenetic modulation of PAG^{GlyT2} cells. Individual animals indicated (n= 5-8/group; mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Table 1: Statistical analysis for all results

Figure	Experiment	Sex	n/group	mean +/- SEM	Test	Main effect	F (DFn, DFd)	*p* value	Post-hoc	Multiple comparison	*p* value
5B	gcamp-vonfrey	Male	4 mice		RM two-way ANOVA with Geisser-Greenhouse correction	Time	F (1.000, 3.000) = 9.916	P=0.0513	Bonferroni	Post: 0.16g vs 2g	>0.9999
						Treatment	F (1.271, 3.813) = 0.5058	P=0.5613		Post: 0.16g vs 6g	>0.9999
						Time x Treatment	F (1.647, 4.942) = 0.6882	P=0.5180		Post: 2g vs 6g	0.6546
										Pre-Post:0.16g	0.1749
										Pre-Post:2g	0.0614
										Pre-Post:6g	0.3773
5C	gcamp-acetone	Male	6 mice	Pre (1.957 ± 0.4205), Post (5.138 ± 0.8184)	two-tailed, paired t-test		t=3.749, df=5	0.0133			

5D	gcamp-hotplate non noxious vs noxious temp	Male	4 mice	31-42 deg (59.74 ± 15.64), 42-50deg (102.2 ± 47.86)	two-tailed, paired t-test		t=0.9939, df=3	0.3935			
5E	gcamp-hotplate rears and jumps	Male	4 mice		RM two-way ANOVA with Geisser-Greenhouse correction	Time	F (1, 3) = 30.53	P=0.0117	Bonferroni	Pre-Post: Rears	0.0072
						Behaviour	F (1, 3) = 5.886	P=0.0937		Pre-Post: Jumps	0.0023
						Time x Behaviour	F (1, 3) = 7.802	P=0.0682			
5F	gcamp-footshock	Male	4 mice		RM two-way ANOVA with Geisser-Greenhouse correction	Time	F (1.000, 3.000) = 183.3	P=0.0009	Bonferroni	Pre-Post: 0.05mA	0.1594
						Treatment	F (1.538, 4.614) = 8.978	P=0.0284		Pre-Post: 0.1mA	0.0154
						Time x Treatment	F (1.418, 4.255) = 5.319	P=0.0741		Pre-Post: 0.2mA	0.0062
										Pre-Post: 0.4mA	0.0181

5G	gcamp-audio tone	Male	4 mice	Pre (1.845 ± 0.4683), Post (10.69 ± 1.588)	two-tailed, paired t-test		t=6.653, df=3	0.0069			
5H	gcamp-claps	Male	5 mice	Pre (2.703 ± 0.4875), Post (3.77 ± 0.6125)	two-tailed, paired t-test		t=5.640, df=4	0.0049			
5I	gcamp-tail grab AUC	Male	5 mice	Pre (-0.06219 ± 0.007365), Post (21.20 ± 4.336)	two-tailed, paired t-test		t=4.896, df=4	0.0081			
6B	vonfrey-control	Male	10 mice	Baseline (1.204 ± 0.2498), Vehicle (0.6940 ± 0.1237), CNO (0.6800 ± 0.1149)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.205, 10.84) = 3.088	P=0.1026	Bonferroni	Baseline vs Vehicle	0.1853
						Individual	F (9, 18) = 1.149	P=0.3814		Baseline vs CNO	0.3886
										Vehicle vs CNO	>0.9999

	vonfrey-excitatory	Male	19 mice	Baseline (1.141 ± 0.1635), Vehicle (0.6315 ± 0.1009), CNO (0.8670 ± 0.1619)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.554, 29.53) = 3.164	P=0.0682	Bonferroni	Baseline vs Vehicle	0.095
						Individual	F (19, 38) = 1.071	P=0.4141		Baseline vs CNO	0.7783
										Vehicle vs CNO	0.3243
	vonfrey-inhibitory	Male	15 mice	Baseline (1.175 ± 0.1750), Vehicle (0.6627 ± 0.1831), CNO (0.8933 ± 0.1249)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.567, 21.94) = 4.049	P=0.0404	Bonferroni	Baseline vs Vehicle	0.1025
						Individual	F (14, 28) = 2.910	P=0.0078		Baseline vs CNO	0.165
										Vehicle vs CNO	0.6497
6C	acetone-control	Male	10 mice	Baseline (3.15 ± 0.422), Vehicle (3.65	RM one-way ANOVA with Geisser-	Treatment	F (1.860, 16.74) = 1.305	P=0.2952	Bonferroni	Baseline vs Vehicle	>0.9999

			± 0.4947), CNO (4.3 ± 0.6464)	Greenhouse correction						
					Individual	F (9, 18) = 1.300	P=0.3028		Baseline vs CNO	0.5576
									Vehicle vs CNO	>0.9999
acetone-excitatory	Male	19 mice	Baseline (3.053 ± 0.1946), Vehicle (3.579 ± 0.3065), CNO (6.158 ± 0.5044)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.473, 26.51) = 25.45	P<0.0001	Bonferroni	Baseline vs Vehicle	0.3699
					Individual	F (18, 36) = 1.560	P=0.1257		Baseline vs CNO	<0.0001
									Vehicle vs CNO	0.0009
acetone-inhibitory	Male	15 mice	Baseline (3.867 ± 0.3065), Vehicle (4.1 ± 0.2193), CNO (1.867 ± 0.3104)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.977, 27.67) = 24.08	P<0.0001	Bonferroni	Baseline vs Vehicle	>0.9999

						Individual	F (14, 28) = 1.809	P=0.0885		Baseline vs CNO	0.0002
										Vehicle vs CNO	<0.0001
6D	hotplate-control	Male	10 mice	Baseline (15.06 ± 0.8993), Vehicle (15.76 ± 1.111), CNO (15.84 ± 0.9364)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.769, 15.92) = 0.3595	P=0.6782	Bonferroni	Baseline vs Vehicle	>0.9999
						Individual	F (9, 18) = 3.628	P=0.0096		Baseline vs CNO	>0.9999
										Vehicle vs CNO	>0.9999
	hotplate-excitatory	Male	20 mice	Baseline (14.42 ± 0.8195), Vehicle (14.93 ± 0.8449), CNO (9.277 ± 0.5998)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.753, 33.30) = 40.15	P<0.0001	Bonferroni	Baseline vs Vehicle	>0.9999
						Individual	F (19, 38) = 5.171	P<0.0001		Baseline vs CNO	<0.0001
										Vehicle vs CNO	<0.0001

	hotplate-inhibitory	Male	15 mice	Baseline (16.1 ± 0.7727), Vehicle (17.49 ± 0.5234), CNO (21.39 ± 1.31)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.753, 24.54) = 11.13	P=0.0006	Bonferroni	Baseline vs Vehicle	0.4721
						Individual	F (14, 28) = 1.828	P=0.0848		Baseline vs CNO	0.0018
										Vehicle vs CNO	0.0313
6E	open field - time mobile-control	Male	10 mice	Vehicle (810.9 ± 48.75), CNO (901.2 ± 34.55)	two-tailed, paired t-test		t=1.677, df=9	0.1279			
	open field - time mobile-excitatory	Male	18 mice	Vehicle (872.2 ± 36.47), CNO (988.1 ± 17.45)	two-tailed, paired t-test		t=2.907, df=17	0.0098			
	open field - time mobile-inhibitory	Male	14 mice	Vehicle (846.5 ± 34.03), CNO (713.7 ± 69.24)	two-tailed, paired t-test		t=2.243, df=13	0.0429			

6F	open field-no. of entries into centre zone-control	Male	10 mice	Vehicle (60.2 ± 9.471), CNO (70.2 ± 7.16)	two-tailed, paired t-test	t=1.309, df=9	0.2229
	open field-no. of entries into centre zone-excitatory	Male	18 mice	Vehicle (66 ± 7.429), CNO (80 ± 4.57)	two-tailed, paired t-test	t=1.765, df=17	0.0955
	open field-no. of entries into centre zone-inhibitory	Male	14 mice	Vehicle (53.71 ± 4.229), CNO (45.07 ± 5.435)	two-tailed, paired t-test	t=1.411, df=13	0.1819
6G	open field-no. rears-control	Male	10 mice	Vehicle (100.3 ± 7.678), CNO (124 ± 14.34)	two-tailed, paired t-test	t=1.406, df=9	0.1934

	open field-no. rears-excitatory	Male	18 mice	Vehicle (105.3 ± 5.147), CNO (100.8 ± 8.143)	two-tailed, paired t-test		t=0.5387, df=17	0.5971			
	open field-no. rears-inhibitory	Male	14 mice	Vehicle (120.4 ± 7.022), CNO (94.43 ± 11.14)	two-tailed, paired t-test		t=2.634, df=13	0.0206			
6H	open field - rears five min interval - control	Male	10 mice		RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (1.710, 15.39) = 2.666	P=0.1073	Bonferroni	Vehicle - CNO: 0-5mins	0.981
						Treatment	F (1.000, 9.000) = 1.976	P=0.1934		Vehicle - CNO: 5-10mins	>0.9999
						Time x Treatment	F (2.635, 23.72) = 1.829	P=0.1741		Vehicle - CNO: 10-15mins	>0.9999
										Vehicle - CNO: 15-20mins	0.0967

open field - rears five min interval - excitatory	Male	18 mice	RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (2.166, 36.83) = 11.48	P<0.0001	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999
				Treatment	F (1.000, 17.00) = 0.5423	P=0.4715		Vehicle - CNO: 5-10mins	>0.9999
				Time x Treatment	F (2.475, 42.07) = 4.461	P=0.0121		Vehicle - CNO: 10-15mins	0.8164
								Vehicle - CNO: 15-20mins	0.1487
open field - rears five min interval - inhibitory	Male	14 mice	RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (2.118, 27.53) = 7.888	P=0.0017	Bonferroni	Vehicle - CNO: 0-5mins	0.025
				Treatment	F (1.000, 13.00) = 6.937	P=0.0206		Vehicle - CNO: 5-10mins	0.4352
				Time x Treatment	F (2.377, 30.90) = 1.224	P=0.3128		Vehicle - CNO: 10-15mins	0.515
								Vehicle - CNO: 15-20mins	>0.9999

6I	open field- no. of jumps- control	Male	10 mice	Vehicle (3 ± 1.751), CNO (4.4 ± 1.492)	two-tailed, paired t-test		t=0.7220, df=9	0.4886			
	open field- no. of jumps- excitatory	Male	18 mice	Vehicle (2.444 ± 1.714), CNO (21.72 ± 4.402)	two-tailed, paired t-test		t=4.612, df=17	0.0002			
	open field- no. of jumps- inhibitory	Male	14 mice	Vehicle (6 ± 1.967), CNO (1.286 ± 0.6498)	two-tailed, paired t-test		t=2.528, df=13	0.0252			
6J	open field - jumps five min interval - control	Male	10 mice		RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (2.199, 19.79) = 1.353	P=0.2831	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999
						Treatment	F (1.000, 9.000) = 0.5213	P=0.4886		Vehicle - CNO: 5-10mins	>0.9999
						Time x Treatment	F (1.714, 15.42) = 0.1269	P=0.8519		Vehicle - CNO: 10-15mins	>0.9999

								Vehicle - CNO: 15-20mins	>0.9999
open field - jumps five min interval - excitatory	Male	18 mice	RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (2.458, 41.79) = 5.358	P=0.0054	Bonferroni	Vehicle - CNO: 0-5mins	0.6565
				Treatment	F (1.000, 17.00) = 21.27	P=0.0002		Vehicle - CNO: 5-10mins	0.0259
				Time x Treatment	F (2.703, 45.95) = 4.552	P=0.0089		Vehicle - CNO: 10-15mins	0.0043
								Vehicle - CNO: 15-20mins	0.0055
open field - jumps five min interval - inhibitory	Male	14 mice	RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (2.396, 31.15) = 2.622	P=0.0796	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999
				Treatment	F (1.000, 13.00) = 6.391	P=0.0252		Vehicle - CNO: 5-10mins	>0.9999
				Time x Treatment	F (1.955, 25.42) = 1.301	P=0.2893		Vehicle - CNO: 10-15mins	0.1576

							Vehicle - CNO: 15-20mins	0.3835
7A	light dark- no. of entries into light zone- control	Male	12 mice	Vehicle (14.83 ± 1.26), CNO (12.17 ± 1.079)	two-tailed, paired t-test	t=1.490, df=11		0.1643
	light dark- no. of entries into light zone- excitatory	Male	13 mice	Vehicle (17.38 ± 1.426), CNO (12.77 ± 1.292)	two-tailed, paired t-test	t=2.511, df=12		0.0274
	light dark- no. of entries into light zone- inhibitory	Male	10 mice	Vehicle (17.5 ± 1.31), CNO (12.2 ± 1.548)	two-tailed, paired t-test	t=2.132, df=9		0.0618
7B	light dark- time spent in light zone- control	Male	12 mice	Vehicle (110.8 ± 8.723), CNO (96.16 ± 7.056)	two-tailed, paired t-test	t=1.330, df=11		0.2105

	light dark-time spent in light zone-excitatory	Male	13 mice	Vehicle (104.2 ± 8.266), CNO (124.7 ± 4.962)	two-tailed, paired t-test	t=2.301, df=12	0.0401
	light dark-time spent in light zone-inhibitory	Male	10 mice	Vehicle (109.1 ± 7.483), CNO (75.43 ± 10.57)	two-tailed, paired t-test	t=3.193, df=9	0.011
7C	light dark-latency to enter dark zone-control	Male	12 mice	Vehicle (2.208 ± 0.3743), CNO (3.1 ± 0.493)	two-tailed, paired t-test	t=1.734, df=11	0.1108
	light dark-latency to enter dark zone-excitatory	Male	13 mice	Vehicle (2.192 ± 0.293), CNO (4.285 ± 0.7312)	two-tailed, paired t-test	t=2.542, df=12	0.0259
	light dark-latency to enter dark	Male	10 mice	Vehicle (2.64 ± 0.423), CNO (2.98 ± 0.6767)	two-tailed, paired t-test	t=0.4172, df=9	0.6863

	zone-inhibitory						
7D	elevated plus maze-no. of entries into open arms-control	Male	6 mice	Vehicle (14.5 ± 3.81), CNO (23.17 ± 4.7)	two-tailed, paired t-test	t=2.225, df=5	0.0767
	elevated plus maze-no. of entries into open arms-excitatory	Male	4 mice	Vehicle (23 ± 3.764), CNO (32.25 ± 2.72)	two-tailed, paired t-test	t=2.846, df=3	0.0653
	elevated plus maze-no. of entries into open arms-inhibitory	Male	6 mice	Vehicle (26.83 ± 1.424), CNO (18.5 ± 1.335)	two-tailed, paired t-test	t=3.217, df=5	0.0235

7E	elevated plus maze-time spent in open arms-control	Male	6 mice	Vehicle (35.22 ± 11.73), CNO (39.68 ± 6.292)	two-tailed, paired t-test		t=0.3525, df=5	0.7388		
	elevated plus maze-time spent in open arms-excitatory	Male	4 mice	Vehicle (53 ± 4.862), CNO (72.05 ± 13.86)	two-tailed, paired t-test		t=1.458, df=3	0.2409		
	elevated plus maze-time spent in open arms-inhibitory	Male	6 mice	Vehicle (61.38 ± 8.164), CNO (29.45 ± 5.42)	two-tailed, paired t-test		t=3.662, df=5	0.0146		
8B	conditioned place aversion day12-%time spent in	Male/Female	13 mice (10 male, 3 female)/group		RM two-way ANOVA with Geisser-Greenhouse correction	Treatment x Time	F (2, 36) = 3.265	P=0.0498	Bonferroni	Pre-Post:mCherry >0.9999

	CNO-paired zone									
					Treatment	F (2, 36) = 0.2898	P=0.7502		Pre-Post:hM3D	0.4743
					Time	F (1, 36) = 3.760	P=0.0604		Pre-Post:hM4D	0.0282
					Subject	F (36, 36) = 2.727	P=0.0017			
8C	conditioned place aversion day12-difference score (cno-veh)	Male/Female	13 mice (10 male, 3 female)/group	RM two-way ANOVA with Geisser-Greenhouse correction	Treatment x Time	F (2, 36) = 3.001	P=0.0623	Bonferroni	Pre-Post:mCherry	>0.9999
					Treatment	F (2, 36) = 0.3630	P=0.6981		Pre-Post:hM3D	0.5668
					Time	F (1, 36) = 3.562	P=0.0672		Pre-Post:hM4D	0.0328
					Subject	F (36, 36) = 2.546	P=0.0031			

8D	conditioned place aversion day12-difference score (post-pre)	Male/Female	13 mice (10 male, 3 female)/group	One-way ANOVA	Treatment	F (2, 36) = 3.265	P=0.0498	Bonferroni	mCherry vs. hM3D	0.3524
									mCherry vs. hM4D	0.0484
									hM3D vs. hM4D	>0.9999
8E	conditioned place aversion day18-%time spent in CNO-paired zone	Male/Female	13 mice (10 male, 3 female)/group	RM two-way ANOVA with Geisser-Greenhouse correction	Treatment x Time	F (2, 36) = 2.795	P=0.0744	Bonferroni	Pre-Post:mCherry	>0.9999
					Treatment	F (2, 36) = 0.3161	P=0.7310		Pre-Post:hM3D	>0.9999
					Time	F (1, 36) = 6.052	P=0.0188		Pre-Post:hM4D	0.006
					Subject	F (36, 36) = 1.863	P=0.0329			

8F	conditioned place aversion day18-difference score (cno-veh)	Male/Female	13 mice (10 male, 3 female)/group	RM two-way ANOVA with Geisser-Greenhouse correction	Treatment x Time	F (2, 36) = 2.730	P=0.0787	Bonferroni	Pre-Post:mCherry	>0.9999
					Treatment	F (2, 36) = 0.3408	P=0.7135		Pre-Post:hM3D	>0.9999
					Time	F (1, 36) = 5.903	P=0.0202		Pre-Post:hM4D	0.0065
					Subject	F (36, 36) = 1.785	P=0.0432			
8G	conditioned place aversion day18-difference score (post-pre)	Male/Female	13 mice (10 male, 3 female)/group	One-way ANOVA	Treatment	F (2, 36) = 2.795	P=0.0744	Bonferroni	mCherry vs. hM3D	>0.9999
									mCherry vs. hM4D	0.1079
									hM3D vs. hM4D	0.2028
S1-A	unilateral vs bilateral-acetone-control	Male	4 unilateral, 6 bilateral	RM two-way ANOVA with Geisser-	Injection x Treatment	F (2, 16) = 3.538	P=0.0534	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999

			Greenhouse correction						
				Injection	F (1, 8) = 3.920	P=0.0831		Unilateral-Bilateral: Vehicle	0.1636
				Treatment	F (2, 16) = 0.8954	P=0.4279		Unilateral-Bilateral: CNO	0.0584
				Subject	F (8, 16) = 1.258	P=0.3299			
unilateral vs bilateral-acetone-excitatory	Male	9 unilateral, 10 bilateral	RM two-way ANOVA with Geisser-Greenhouse correction	Injection x Treatment	F (2, 34) = 0.9568	P=0.3942	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999
				Injection	F (1, 17) = 0.01656	P=0.8991		Unilateral-Bilateral: Vehicle	0.8313
				Treatment	F (2, 34) = 25.07	P<0.0001		Unilateral-Bilateral: CNO	>0.9999
				Subject	F (17, 34) = 1.646	P=0.1059			
unilateral vs bilateral-acetone-inhibitory	Male	4 unilateral, 11 bilateral	RM two-way ANOVA with Geisser-	Injection x Treatment	F (2, 26) = 4.182	P=0.0266	Bonferroni	Unilateral-Bilateral: Baseline	0.1894

				Greenhouse correction						
					Injection	F (1, 13) = 0.001726	P=0.9675		Unilateral-Bilateral: Vehicle	0.7608
					Treatment	F (2, 26) = 21.00	P<0.0001		Unilateral-Bilateral: CNO	>0.9999
					Subject	F (13, 26) = 2.391	P=0.0284			
S1-B	unilateral vs bilateral-hotplate-control	Male	4 unilateral, 6 bilateral	RM two-way ANOVA with Geisser-Greenhouse correction	Injection x Treatment	F (2, 16) = 0.5932	P=0.5643	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999
					Injection	F (1, 8) = 0.3537	P=0.5685		Unilateral-Bilateral: Vehicle	>0.9999
					Treatment	F (2, 16) = 0.1929	P=0.8265		Unilateral-Bilateral: CNO	0.9278
					Subject	F (8, 16) = 3.710	P=0.0123			
	unilateral vs bilateral-hotplate-excitatory	Male	9 unilateral, 10 bilateral	RM two-way ANOVA with Geisser-	Injection x Treatment	F (2, 34) = 0.4328	P=0.6522	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999

				Greenhouse correction						
					Injection	F (1, 17) = 0.1317	P=0.7212		Unilateral-Bilateral: Vehicle	>0.9999
					Treatment	F (2, 34) = 34.80	P<0.0001		Unilateral-Bilateral: CNO	>0.9999
					Subject	F (17, 34) = 5.433	P<0.0001			
	unilateral vs bilateral-hotplate-inhibitory	Male	4 unilateral, 11 bilateral	RM two-way ANOVA with Geisser-Greenhouse correction	Injection x Treatment	F (2, 26) = 1.945	P=0.1632	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999
					Injection	F (1, 13) = 0.8419	P=0.3756		Unilateral-Bilateral: Vehicle	>0.9999
					Treatment	F (2, 26) = 6.433	P=0.0054		Unilateral-Bilateral: CNO	0.1702
					Subject	F (13, 26) = 1.978	P=0.0674			
S1-C	unilateral vs bilateral-acetone-control	Female	5 unilateral, 3 bilateral	RM two-way ANOVA with Geisser-	Injection x Treatment	F (2, 12) = 0.1018	P=0.9040	Bonferroni	Unilateral-Bilateral: Baseline	0.4846

			Greenhouse correction						
				Injection	F (1, 6) = 7.427	P=0.0344		Unilateral-Bilateral: Vehicle	0.359
				Treatment	F (2, 12) = 2.969	P=0.0896		Unilateral-Bilateral: CNO	0.1654
				Subject	F (6, 12) = 1.311	P=0.3238			
unilateral vs bilateral-acetone-excitatory	Female	4 unilateral, 8 bilateral	RM two-way ANOVA with Geisser-Greenhouse correction	Injection x Treatment	F (2, 20) = 0.09371	P=0.9109	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999
				Injection	F (1, 10) = 0.3147	P=0.5872		Unilateral-Bilateral: Vehicle	>0.9999
				Treatment	F (2, 20) = 8.126	P=0.0026		Unilateral-Bilateral: CNO	>0.9999
				Subject	F (10, 20) = 2.084	P=0.0778			
unilateral vs bilateral-acetone-inhibitory	Female	4 unilateral, 7 bilateral	RM two-way ANOVA with Geisser-	Injection x Treatment	F (2, 18) = 2.973	P=0.0766	Bonferroni	Unilateral-Bilateral: Baseline	0.2053

				Greenhouse correction						
					Injection	F (1, 9) = 0.6357	P=0.4458		Unilateral-Bilateral: Vehicle	>0.9999
					Treatment	F (2, 18) = 2.795	P=0.0877		Unilateral-Bilateral: CNO	>0.9999
					Subject	F (9, 18) = 3.642	P=0.0094			
S1-D	unilateral vs bilateral-hotplate-control	Female	5 unilateral, 3 bilateral	RM two-way ANOVA with Geisser-Greenhouse correction	Injection x Treatment	F (2, 12) = 1.901	P=0.1917	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999
					Injection	F (1, 6) = 2.263	P=0.1832		Unilateral-Bilateral: Vehicle	>0.9999
					Treatment	F (2, 12) = 3.922	P=0.0489		Unilateral-Bilateral: CNO	0.1015
					Subject	F (6, 12) = 2.140	P=0.1235			
	unilateral vs bilateral-hotplate-excitatory	Female	4 unilateral, 8 bilateral	RM two-way ANOVA with Geisser-	Injection x Treatment	F (2, 20) = 5.612	P=0.0116	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999

			Greenhouse correction						
				Injection	F (1, 10) = 2.348	P=0.1565		Unilateral-Bilateral: Vehicle	0.0098
				Treatment	F (2, 20) = 0.3323	P=0.7212		Unilateral-Bilateral: CNO	>0.9999
				Subject	F (10, 20) = 1.893	P=0.1078			
unilateral vs bilateral-hotplate-inhibitory	Female	4 unilateral, 7 bilateral	RM two-way ANOVA with Geisser-Greenhouse correction	Injection x Treatment	F (2, 18) = 2.028	P=0.1606	Bonferroni	Unilateral-Bilateral: Baseline	>0.9999
				Injection	F (1, 9) = 2.847	P=0.1258		Unilateral-Bilateral: Vehicle	0.7997
				Treatment	F (2, 18) = 6.450	P=0.0077		Unilateral-Bilateral: CNO	0.061
				Subject	F (9, 18) = 5.222	P=0.0014			

S5-B	vonfrey-control	Female	6 mice	Baseline (0.8833 ± 0.06667), Vehicle (0.7283 ± 0.1721), CNO (0.5533 ± 0.1320)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.289, 6.447) = 1.140	P=0.3449	Bonferroni	Baseline vs Vehicle	>0.9999
						Individual	F (5, 10) = 0.1523	P=0.9746		Baseline vs CNO	0.2268
										Vehicle vs CNO	>0.9999
	vonfrey-excitatory	Female	8 mice	Baseline (0.8675 ± 0.08250), Vehicle (0.9725 ± 0.2991), CNO (0.6825 ± 0.1873)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.443, 10.10) = 0.4197	P=0.6046	Bonferroni	Baseline vs Vehicle	>0.9999
						Individual	F (7, 14) = 0.5569	P=0.7783		Baseline vs CNO	>0.9999
										Vehicle vs CNO	>0.9999

	vonfrey-inhibitory	Female	8 mice	Baseline (0.8500 ± 0.3017), Vehicle (0.6863 ± 0.1696), CNO (1.401 ± 0.5388)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.772, 12.40) = 1.861	P=0.1978	Bonferroni	Baseline vs Vehicle	>0.9999
						Individual	F (7, 14) = 3.439	P=0.0235		Baseline vs CNO	0.533
										Vehicle vs CNO	0.4708
S5-C	acetone-control	Female	8 mice	Baseline (4.375 ± 0.4407), Vehicle (5.813 ± 0.6404), CNO (4.563 ± 0.4766)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.219, 8.533) = 3.588	P=0.0879	Bonferroni	Baseline vs Vehicle	0.2737
						Individual	F (7, 14) = 2.885	P=0.0434		Baseline vs CNO	>0.9999
										Vehicle vs CNO	0.2677

	acetone-excitatory	Female	12 mice	Baseline (3.917 ± 0.2525), Vehicle (4.083 ± 0.237), CNO (5.583 ± 0.4557)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.415, 15.57) = 10.62	P=0.0026	Bonferroni	Baseline vs Vehicle	>0.9999
						Individual	F (11, 22) = 2.130	P=0.0632		Baseline vs CNO	0.0091
										Vehicle vs CNO	0.0266
	acetone-inhibitory	Female	11 mice	Baseline (4.091 ± 0.329), Vehicle (4.045 ± 0.4181), CNO (3.318 ± 0.3585)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.988, 19.88) = 2.252	P=0.1315	Bonferroni	Baseline vs Vehicle	>0.9999
						Individual	F (10, 20) = 2.931	P=0.0195		Baseline vs CNO	0.2372
										Vehicle vs CNO	0.3489
S5-D	hotplate-control	Female	8 mice	Baseline (13.02 ± 0.7071), Vehicle	RM one-way ANOVA with Geisser-	Treatment	F (1.763, 12.34) = 2.580	P=0.1200	Bonferroni	Baseline vs Vehicle	>0.9999

			(12.73 ± 0.7398), CNO (15.07 ± 1.272)	Greenhouse correction						
					Individual	F (7, 14) = 2.238	P=0.0946		Baseline vs CNO	0.3338
									Vehicle vs CNO	0.3362
hotplate-excitatory	Female	12 mice	Baseline (14.35 ± 0.7732), Vehicle (14.06 ± 1.286), CNO (13.01 ± 1.313)	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.350, 14.85) = 0.4404	P=0.5757	Bonferroni	Baseline vs Vehicle	>0.9999
					Individual	F (11, 22) = 1.495	P=0.2029		Baseline vs CNO	>0.9999
									Vehicle vs CNO	>0.9999
hotplate-inhibitory	Female	11 mice	Baseline (14.6 ± 1.283), Vehicle (15.46 ± 1.327), CNO	RM one-way ANOVA with Geisser-Greenhouse correction	Treatment	F (1.236, 12.36) = 8.397	P=0.0100	Bonferroni	Baseline vs Vehicle	>0.9999

				(20.76 ± 2.582)						
						Individual	F (10, 20) = 5.606	P=0.0005	Baseline vs CNO	0.0432
									Vehicle vs CNO	0.0316
S5-E	open field - time mobile- control	Female	5 mice	Vehicle (722.4 ± 64.64), CNO (733.4 ± 54.99)	two-tailed, paired t-test		t=0.1798, df=4	0.8661		
	open field - time mobile- excitatory	Female	11 mice	Vehicle (698.8 ± 44.33), CNO (932.3 ± 47.87)	two-tailed, paired t-test		t=2.888, df=10	0.0162		
	open field- time mobile- inhibitory	Female	10 mice	Vehicle (797.1 ± 40.12), CNO (615.7 ± 93.09)	two-tailed, paired t-test		t=2.034, df=9	0.0724		

S5-F	open field-no. of entries into centre zone-control	Female	5 mice	Vehicle (58.2 ± 8.924), CNO (50.2 ± 9.759)	two-tailed, paired t-test	t=0.7638, df=4	0.4875
	open field-no. of entries into centre zone-excitatory	Female	11 mice	Vehicle (46.82 ± 5.072), CNO (65.91 ± 6.146)	two-tailed, paired t-test	t=2.561, df=10	0.0283
	open field-no. of entries into centre zone-inhibitory	Female	10 mice	Vehicle (55.8 ± 7.47), CNO (46.7 ± 9.953)	two-tailed, paired t-test	t=0.9269, df=9	0.3782
S5-G	open field-no. of rears-control	Female	5 mice	Vehicle (107.4 ± 14.83), CNO (103 ± 19.5)	two-tailed, paired t-test	t=0.2875, df=4	0.788

	open field-no. of rears-excitatory	Female	11 mice	Vehicle (108.1 ± 10.13), CNO (105.6 ± 13.81)	two-tailed, paired t-test		t=0.1179, df=10	0.9085			
	open field-no. of rears-inhibitory	Female	10 mice	Vehicle (112.3 ± 9.177), CNO (78.3 ± 15.16)	two-tailed, paired t-test		t=2.396, df=9	0.0402			
S5-H	open field-rears five min interval-control	Female	5 mice		RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (2.020, 8.082) = 1.847	P=0.2184	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999
						Treatment	F (1.000, 4.000) = 0.08264	P=0.7880		Vehicle - CNO: 5-10mins	>0.9999
						Time x Treatment	F (1.293, 5.173) = 0.6243	P=0.5050		Vehicle - CNO: 10-15mins	>0.9999
										Vehicle - CNO: 15-20mins	>0.9999
	open field-rears five min	Female	11 mice		RM two-way ANOVA with Geisser-	Time interval	F (1.966, 19.66) = 4.634	P=0.0229	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999

interval-excitatory			Greenhouse correction					Vehicle - CNO: 5-10mins	>0.9999
				Treatment	F (1.000, 10.00) = 0.01389	P=0.9085		Vehicle - CNO: 10-15mins	>0.9999
				Time x Treatment	F (2.834, 28.34) = 0.5277	P=0.6570		Vehicle - CNO: 15-20mins	>0.9999
open field-rears five min interval-inhibitory	Female	10 mice	RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (1.627, 14.64) = 4.587	P=0.0344	Bonferroni	Vehicle - CNO: 0-5mins	0.6021
				Treatment	F (1.000, 9.000) = 5.740	P=0.0402		Vehicle - CNO: 5-10mins	0.3677
				Time x Treatment	F (2.147, 19.33) = 0.07843	P=0.9350		Vehicle - CNO: 10-15mins	0.362
								Vehicle - CNO: 15-20mins	0.106

S5-I	open field-no. of jumps-control	Female	5 mice	Vehicle (4 ± 1.673), CNO (5.2 ± 2.746)	two-tailed, paired t-test		t=0.5632, df=4	0.6034			
	open field-no. of jumps-excitatory	Female	11 mice	Vehicle (0.5455 ± 0.3659), CNO (8.273 ± 4.132)	two-tailed, paired t-test		t=1.867, df=10	0.0914			
	open field-no. of jumps-inhibitory	Female	10 mice	Vehicle (1.9 ± 0.9363), CNO (2.1 ± 0.9826)	two-tailed, paired t-test		t=0.4523, df=9	0.6618			
S5-J	open field-jumps five min interval-control	Female	5 mice		RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (1.702, 6.808) = 1.422	P=0.2986	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999
						Treatment	F (1.000, 4.000) = 0.3172	P=0.6034		Vehicle - CNO: 5-10mins	>0.9999
						Time x Treatment	F (1.764, 7.057) = 0.3642	P=0.6824		Vehicle - CNO: 10-15mins	0.2819

								Vehicle - CNO: 15-20mins	>0.9999
open field-jumps five min interval-excitatory	Female	11 mice	RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (1.586, 15.86) = 0.3202	P=0.6812	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999
				Treatment	F (1.000, 10.00) = 3.486	P=0.0914		Vehicle - CNO: 5-10mins	0.2622
				Time x Treatment	F (1.790, 17.90) = 0.4383	P=0.6304		Vehicle - CNO: 10-15mins	0.2837
								Vehicle - CNO: 15-20mins	0.276
open field-jumps five min interval-inhibitory	Female	10 mice	RM two-way ANOVA with Geisser-Greenhouse correction	Time interval	F (1.242, 11.18) = 0.07045	P=0.8463	Bonferroni	Vehicle - CNO: 0-5mins	>0.9999
				Treatment	F (1.000, 9.000) = 0.2045	P=0.6618		Vehicle - CNO: 5-10mins	0.2704
				Time x Treatment	F (1.162, 10.46) = 2.372	P=0.1521		Vehicle - CNO: 10-15mins	0.3245

							Vehicle - CNO: 15-20mins	0.7737
S6-A	light dark- no. of entries into light zone- control	Female	6 mice	Vehicle (15.17 ± 2.701), CNO (17.67 ± 1.498)	two-tailed, paired t-test	t=1.263, df=5	0.2622	
	light dark- no. of entries into light zone- excitatory	Female	8 mice	Vehicle (16.5 ± 2.079), CNO (12.63 ± 1.625)	two-tailed, paired t-test	t=1.609, df=7	0.1517	
	light dark- no. of entries into light zone- inhibitory	Female	7 mice	Vehicle (16.71 ± 1.554), CNO (11.43 ± 2.148)	two-tailed, paired t-test	t=1.598, df=6	0.1611	
S6-B	light dark- time spent in light zone- control	Female	6 mice	Vehicle (84.7 ± 13.4), CNO (96.35 ± 13.94)	two-tailed, paired t-test	t=1.060, df=5	0.3376	

	light dark-time spent in light zone-excitatory	Female	8 mice	Vehicle (100.3 ± 9.689), CNO (130.9 ± 5.149)	two-tailed, paired t-test	t=4.352, df=7	0.0033
	light dark-time spent in light zone-inhibitory	Female	7 mice	Vehicle (102.3 ± 11.63), CNO (80.91 ± 18.11)	two-tailed, paired t-test	t=1.772, df=6	0.1267
S6-C	light dark-latency to enter dark zone-control	Female	6 mice	Vehicle (2.533 ± 0.9587), CNO (3.85 ± 0.7706)	two-tailed, paired t-test	t=1.152, df=5	0.3015
	light dark-latency to enter dark zone-excitatory	Female	8 mice	Vehicle (1.813 ± 0.2125), CNO (5.375 ± 2.407)	two-tailed, paired t-test	t=1.421, df=7	0.1984
	light dark-latency to enter dark	Female	7 mice	Vehicle (3.814 ± 0.8433), CNO	two-tailed, paired t-test	t=0.6017, df=6	0.5694

zone- inhibitory	(3.057 ± 0.7508)