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Preproenkephalin-expressing ventral pallidal neurons control inhibitory avoidance learning

Tom Macpherson[a], Hiroyuki Mizoguchi[b], Akihiro Yamanaka[c], Takatoshi Hikida[a,*]

[a] Laboratory for Advanced Brain Functions, Institute for Protein Research, Osaka University, Japan
[b] Research Center for Next-Generation Drug Development, Research Institute of Environmental Medicine, Nagoya University, Japan
[c] Department of Neuroscience II, Research Institute of Environmental Medicine, Nagoya University, Japan

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ABSTRACT

The ventral pallidum (VP) is a critical component of the basal ganglia neurocircuitry regulating learning and decision making; however, its precise role in controlling associative learning of environmental stimuli conditioned to appetitive or aversive outcomes is still unclear. Here, we investigated the expression of preproenkephalin, a polypeptide hormone previously shown to be expressed in nucleus accumbens neurons controlling aversive learning, within GABAergic and glutamatergic VP neurons. Next, we explored the behavioral consequences of chemicogenetic inhibition or excitation of preproenkephalin-expressing VP neurons on associative learning of reward- or aversion-paired stimuli in autoshaping and inhibitory avoidance tasks, respectively. We reveal for the first time that preproenkephalin is expressed predominantly in GABAergic rather than glutamatergic VP neurons, and that excitation of these preproenkephalin-expressing VP neurons was sufficient to impair inhibitory avoidance learning. These findings indicate the necessity for inhibition of preproenkephalin-expressing VP neurons for avoidance learning, and suggest these neurons as a potential therapeutic target for psychiatric disorders associated with maladaptive aversive learning.

1. Introduction

The ability to learn and respond to reward- and aversive-predictive stimuli in our environment is critical for survival. The basal ganglia nuclei are thought to be crucially involved in these types of learning, and dysfunction of this circuitry can result in several neuropathologies associated with maladaptive or impaired learning, including schizophrenia, drug addiction, and depression (Hikida et al., 2016; Macpherson et al., 2014). Our group has previously demonstrated that nucleus accumbens (NAc) dopamine D1- and D2-receptor-expressing medium spiny neurons (MSNs) contribute to reward and aversive associative learning, respectively (Hikida et al., 2010; Macpherson and Hikida, 2018). However, the role of downstream projection sites of the NAc in controlling reward and aversive learning is still unclear. While classically it was thought that outputs from NAc D1- and D2-MSNs were received predominantly in the substancia nigra pars reticulata (SNr) and ventral pallidum (VP), respectively, recent evidence has shown that approximately half of all NAc D1-MSNs project to the VP alongside the entirety of NAc D2-MSNs (Kupchik et al., 2015). These findings suggest the VP as a possible site of interest in the investigation of the neurocircuitry of associative learning.

The VP is a critical component of the basal ganglia neurocircuitry, and has been proposed to direct "motivation into action" by linking the mesolimbic dopamine system with motor output areas (Mogenson et al., 1980; Root et al., 2015). As well as receiving GABAergic projections from the NAc, the VP is also densely innervated by dopaminergic fibers from the ventral tegmental area (VTA), suggesting that it likely integrates motivationally salient reward/aversive signals (Klitenick et al., 1992; Napier et al., 1991; Napier and Potter, 1989). Indeed, various manipulations of the VP have been shown to alter reward and aversive learning. Intra-VP infusions of GABA receptor agonists reduced the accuracy of both cue-induced responding for a food

Abbreviations: CNO, clozapine n-oxide; CS, conditioned stimulus; DREADD, designer receptor exclusively activated by designer drug; LHb, lateral habenula; MSN, medium spiny neuron; NAc, nucleus accumbens; Penk, Preproenkephalin; RNB, reversible neurotransmission blocking; SNr, substancia nigra pars reticulata; STN, subthalamic nucleus; TeNT, tetanus neurotoxin; TRE, tetracycline-responsive element; tTA, tetracycline-repressive transcription factor; VGAT, vesicular GABA transporter; VGlUT2, vesicular glutamate transporter 2; VTA, ventral tegmental area; VP, ventral pallidum

* Corresponding author. Laboratory for Advanced Brain Functions, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka, 565-0871, Japan.
E-mail address: hikida@protein.osaka-u.ac.jp (T. Hikida).

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reward in a discriminative stimulus task and cue-induced responding in a Pavlovian-to-instrumental transfer task (Leung and Balleine, 2015; Richard et al., 2016). In an aversive learning task, intra-VP infusions of dopamine D1 and D2 agonists increased the latency to enter a shock-paired chamber in an inhibitory avoidance task, indicating that activation of VP D1 or D2 receptors is able to enhance aversive learning (Lénárd et al., 2017; Péczely et al., 2014). More recently, in-vivo single-cell recording of VP neurons in primates during a mixed outcome delayed response task revealed neural activity following presentation of both appetitive and aversive conditioned stimuli (CS) as well as appetitive and aversive outcome anticipatory activity (Saga et al., 2017). Moreover, in the same experiment, intra-VP infusions of a GABA antagonist during the same task impaired the ability to avoid the aversive outcome. Together, these studies indicate that the VP encodes both reward and aversive information, and that disruption of VP activity impairs the ability of specific CS-outcome associations to guide actions.

In situ evidence has shown that the VP contains a significant population of preproenkephalin (Penk)-expressing neurons (Kalivas et al., 1993). Penk is an endogenous opoid polypeptide hormone, which when cleaved results in the generation of the pentapetides [Met]enkephalin and, to a lesser extent, [Leu]enkephalin (Höllt, 1983). Given that Penk-expressing NAc MSNs that project to the VP have been demonstrated to control aversive learning as well as reversal learning in appetitive tasks (Hikida et al., 2010; Macpherson et al., 2016; Yawata et al., 2012), it is possible that Penk-expressing neurons in the VP may also play an important role in reward and aversive learning. However, as of present, it is still unclear in which VP neurons Penk is expressed. Indeed, alongside GABAergic neurons, the VP also contains a large population of glutamatergic neurons as well as a small population of cholinergic neurons (Hur and Zaborszky, 2005; Root et al., 2015; Zaborszky et al., 2012).

Here we began by investigating the expression of Penk within the two major neuron types in the VP, GABAergic and glutamatergic neurons. Then, we targeted VP Penk-expressing neurons using two Tet-Tag AAV virus methods to either inhibit their activity by reversible neurotransmission blocking (RNB) in transgenic mice or stimulate activity in these neurons via the expression of designer receptors exclusively activated by designer drugs (DREADDs). Using these methods, we investigated the behavioral consequences of inhibition or activation of Penk-expressing VP neurons on reward and aversive associative learning during the acquisition of autoshaping and inhibitory avoidance tasks, respectively. In the autoshaping task, presentation of a conditioned stimulus (CS) associated with a reward (CS+), but not a CS associated with no consequence (CS-), typically results in either "sign-tracking" approach behavior to the CS + or "goal-tracking" approach behavior to the location of the reward (i.e. food magazine) (Boakes, 1977; Bussey et al., 1997; Cardinal et al., 2002). Interestingly, these two conditioned responses have been demonstrated to be controlled by distinct neural mechanisms, with activity in dopamine release in the NAc and NAc D1-neuron activation necessary for sign-tracking but not goal-tracking behavior (Flagel et al., 2011; Macpherson and Hikida, 2018). However, the potential role of Penk-expressing VP neurons in the acquisition of Pavlovian approach behavior to a reward-paired conditioned stimulus (sign-tracking) or Pavlovian approach behavior to a primary liquid reward (goal-tracking) is still unclear. Finally, in the inhibitory avoidance task, the delivery of a foot-shock is paired with a specific environment leads to the formation of an aversive Pavlovian association that triggers avoidance behavior upon re-exposure to the same environment (Banfi et al., 1982; Kallman and Condie, 1985).

These experiments revealed a role for Penk-expressing VP neurons in controlling the acquisition of aversive, but not reward-related, associative learning tasks.

2. Materials and methods

2.1. Animals

For reversible neurotransmission blocking (RNB) experiments, male preproenkephalin (Penk)-expressing neuron blocked (Penk-RNB) and wildtype (WT) mice aged between 8 and 10 weeks were generated using tetanus neurotoxin (TeNT) and enhanced green fluorescent protein (EGFP) transgenic mice on a C57BL/6J background, as previously described (Hikida et al., 2010). In TeNT mice, the expression of TeNT, a bacterial toxin that cleaves the synaptic-vesicle-associated VAMP2 protein and abolishes neurotransmitter release from the synaptic vesicles of the target neurons, as well as EGFP, is under the control of tetracycline-responsive element (TRE) and is driven by the binding of TRE with tetracycline-repressive transcription factor (tTA) in the absence of doxycycline (Schiavo et al., 1992; Wada et al., 2007). tTA expression was restricted to Penk-expressing neurons by intra-VP infusion of a recombinant AAV virus (AAV2-Penk-tTA), as described in (Hikida et al., 2010). For DREADD and *in situ* hybridization experiments, male C57BL/6J mice between 8 and 10 weeks of age were used (CLEA Japan Inc, Tokyo, Japan). All mice were housed in groups of 2–3 in cages containing woodchip bedding and cardboard nesting material, and were maintained on a 12-h light/dark cycle (lights on at 8:00 a.m.) with the temperature controlled to 24 ± 2 °C in a humidity of 50 ± 5%.

2.2. Surgical procedures

Following anesthesia (90 mg/kg Ketamine and 20 mg/kg Xylazine, i.p. injection), AAV2-Penk-tTA (500 nl/site at 100 nl/min; left for 5 min) or AAVDJ-TetO (3G)-hM3Dq-mCherry and AAV2-Penk-tTA mixed at a 1:1 ratio (250 nl of each virus/site at 100 nl/min; left for 5 min) were bilaterally injected into the VP (AP +0.0 ML ± 1.5 DV -4.25 and AP +0.6 ML ± 1.25 DV -4.5) of TeNT and WT, and C57BL/6J mice, respectively, to create Penk-RNB and Penk-hM3Dq mice. Mice were left in their home cages for three weeks prior to the start of behavioral experiments to allow sufficient time for recovery and expression of the viruses, during which time they were observed daily for signs of discomfort/disability. Additionally, from birth, and throughout the duration of the experiment, mice were never treated with doxycycline, creating a persistent blockade of neurotransmission in Penk-RNB mice. Following the completion of experiments, virus placements were histologically verified (see Supp Fig. 1A–C), and two mice were excluded from the analysis due to misaligned injection sites. All animal handling procedures and use of viral and tetanus toxin constructs were approved by the animal research committee of the Institute for Protein Research, Osaka University.

2.3. Drugs

Clozapine-N-oxide (CNO; Sigma Aldrich, MO, USA) was dissolved in saline solution containing 0.5% DMSO. For behavior experiments, CNO was injected intraperitoneally (*i.p.*) at 0.1 mg/kg, 10 mL/kg, 30 min before testing, and mice were separated into individual cages until testing.

2.4. Autoshaping

The autoshaping task was performed as previously described (Horner et al., 2013; Macpherson and Hikida, 2018) with minor modification. The autoshaping apparatus consisted of sound- and light-resistant trapezoidal touch-screen operant chambers containing a liquid reward magazine located in the center of a touchscreen panel (Campden Instruments Ltd., UK). Following two consecutive days of a 40-min magazine training session in which they received a 2000 μl 10% condensed milk solution at the start of the session, mice underwent six

consecutive days of a 90-min conditioning session in which the left and right sides of the touch panel were separately illuminated for 10 s with a visual stimulus (white rectangle). One side was repeatedly paired with a 500 µl 10% condensed milk reward and was termed the CS+, while the other side was paired with no reward and was termed the CS-. The CS+ and CS- were each presented 20 times in a randomized order with a 10–40 s variable inter-trial-interval (ITI) between each presentation and the assignment of the CS+/CS- side was counterbalanced among animals. Penk-hM3Dq mice received either i.p. CNO (0.1 mg/kg) or saline 30 min prior to each conditioning session. In accordance with a previously described protocol for the autoshaping task in a touch-screen operant chamber (Horner et al., 2013), the initial response of the mouse during each CS trial was recorded. Therefore, when the mouse broke a photocell beam located 3 cm in front of the CS+/CS- before any entry into the reward magazine a sign-tracking response was registered, whereas when the mouse broke a photocell beam within the reward magazine before any CS+/CS- approach a goal-tracking response was registered.

2.5. Inhibitory avoidance

The inhibitory avoidance task was performed as previously described (Hikida et al., 2010, 2013) with minor modification. The inhibitory avoidance apparatus (Med Associates Inc, VT, USA) consisted of two chambers separated by a black sliding door; a small, light chamber illuminated by a lamp (9.78 cm L x 12.7 cm D x 12.7 cm H) and a large, dark chamber covered with black cloth and containing a grid floor connected to an electric shock source (16.76 cm L x 12.7 cm D x 12.7 cm H). Penk-hM3Dq mice received either i.p. CNO (0.1 mg/kg) or saline 30 min prior to the conditioning session. During the conditioning session, the mouse was placed in the light chamber and the door leading to the dark chamber was raised. When all four paws of the mouse had entered into the dark chamber, the door was closed; and then an electric footshock (0.3 mA, 60 Hz, 1 s) was delivered and the mouse was left in the chamber for 1 min. Inhibitory avoidance learning was tested 24 h later by measuring the latency to step into the dark (shock-paired) chamber.

2.6. Fluorescent in situ hybridization

For analysis of preproenkephalin (Penk), vesicular GABA transporter (VGAT), and vesicular glutamate transporter 2 (VGLUT2) expression within the VP, male C57BL/6J mice were subjected to fluorescent *in situ* hybridization (FISH). For confirmation of Penk-expressing neuron-specific expression of hM3Dq DREADDs within the VP, FISH was performed using Penk-hM3Dq mice treated with CNO (0.1 mg/kg) and left in their homecage two hours prior to dissection of brains. Following cervical dislocation, freshly dissected brains were flash frozen in isopentane (Nacalai Tesque, Kyoto, Japan) and sliced into 10 µm coronal sections on a cryostat. Sections containing the VP were processed by fluorescent *in situ* hybridization (FISH) using RNAscope (Advanced Cell Diagnostics, Hayward, CA, USA) following the manufacturer's protocol with probes against preproenkephalin (RNAscope Probe- Mm-Penk, Cat No. 318761-C2, Advanced Cell Diagnostics, CA, USA) and either VGAT (RNAscope Probe- Mm-Slc32a1, Cat No. 319191, Advanced Cell Diagnostics, CA, USA), VGLUT2 (RNAscope Probe- Mm-Slc17a6, Cat No. 319171, Advanced Cell Diagnostics, CA, USA), mCherry (RNAscope Probe- mCherry*E, Cat No. 404491, Advanced Cell Diagnostics, CA, USA), or Fos (RNAscope Probe- Mm-Fos, Cat No. 316921, Advanced Cell Diagnostics, CA, USA). Finally, cell nuclei were stained with DAPI, then images of the ventral pallidum from slices approximately 0.6 mm and 0 mm to Bregma were captured using a Keyence BZ-X810 fluorescence microscope (Keyence, Osaka, Japan) and an Olympus FV1000D (IX) confocal laser scanning microscope (Olympus, Tokyo, Japan) using 40x objectives. The number of Penk-labeled, VGAT-, VGLUT2-, mCherry-, or Fos-labeled, and double-labeled neurons, as well as the number of DAPI-labeled neurons, were hand counted using ImageJ software with a cell counter plugin by an observer blinded to the probes used in each image. Images were collected from a total of three mice for each area and set of probes, and the total number of positively-labeled neurons for each probe was averaged. The average number of Penk-,VGAT-, VGLUT2-, mCherry-, or Fos-labeled neurons was divided by the average total number of neurons (DAPI-labeled) then multiplied by 100 to give the percentage of total neurons labeled with each probe. The percentage of the total VP neurons double-labeled for two of the probes was calculated using the same method, and the percentage of Penk-expressing VGAT and VGLUT2 neurons was calculated by dividing the average number of double-labeled neurons by the average total number of VGAT or VGLUT2 neurons, respectively, and multiplying by 100.

2.7. Immunofluorescence

After behavioral tests, Penk-RNB mice, as well as Penk-hM3Dq mice treated with CNO (0.1 mg/kg, 10 mL/kg; Sigma-Aldrich, MO, USA) and left in their home cages for 2.5 h, were anesthetized (90 mg/kg Ketamine and 20 mg/kg Xylazine, i.p. injection) and then transcardially perfused with cold 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan). Brains were then dissected and placed in 30% sucrose in PBS for three days until they became completely submerged. Coronal sections were sliced using a cryostat at 40 µm (Leica CM1860, Leica Biosystems, Wetzlar, Germany). Immunohistochemistry was performed (as described (Ohishi et al., 1994)) using the following primary antibodies: mouse monoclonal anti-mCherry (1C51) and rabbit polyclonal anti-GFP (A-11122) (1:500; ThermoFisher Scientific, MA, USA). Fluorescent secondary antibodies conjugated to Alexa 568 or Alexa 488 (Life Technologies, CA, USA) were used, respectively. The sections were mounted with Vectashield containing DAPI (Vector Laboratories, CA, USA) and captured using an Axiocam 503 mono camera (Zeiss, Oberkochen, Germany) attached to a Zeiss Observer Z1 fluorescence microscope (Zeiss, Oberkochen, Germany) and a Keyence BZ-X810 fluorescence microscope (Keyence, Osaka, Japan).

2.8. Data analysis

All data are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) and SPSS 22.0 (IBM Corporation, NY, USA) software. For autoshaping data, the session totals of the initial conditioned response (sign-tracking or goal-tracking) for each of 20 CS+ and 20 CS- presentations were analyzed using three-way repeated measures ANOVAs. For inhibitory avoidance data, statistical differences between the two drug groups before and after conditioning were determined using a two-way repeated-measures ANOVA. *Post hoc* analyses were conducted where appropriate using student's t-tests.

3. Results

3.1. Expression of preproenkephalin in GABAergic ventral pallidal neurons

To investigate the expression of Penk in the VP of wildtype mice, we performed FISH using probes for Penk and either VGAT (Fig 1A&B, n = 3) or VGLUT2 (Fig 1C&D, n = 3), to identify GABAergic and glutamatergic neurons respectively. In the rostral VP, 47.3% ± 3.4 (total: 433 of 916 DAPI-labeled neurons) of neurons were positively labeled for Penk (Figs. 1A&C and 2A), while in the caudal VP, 42.3% ± 6.3 (total: 421 of 995 DAPI-labeled neurons) were positively labeled for Penk (Figs. 1B&D and 2G). Dual-labeling FISH in the rostral VP revealed that 73.2% ± 5.7 (total: 369 of 504 DAPI-labeled neurons) of neurons were positively labeled for VGAT and 46.2% ± 3.4 (total: 233) showed co-labeling of Penk and VGAT (Figs. 1A and 2B&C), while 18.2% ± 0.6 (total: 75 of 412 DAPI-labeled neurons) were positively labeled for

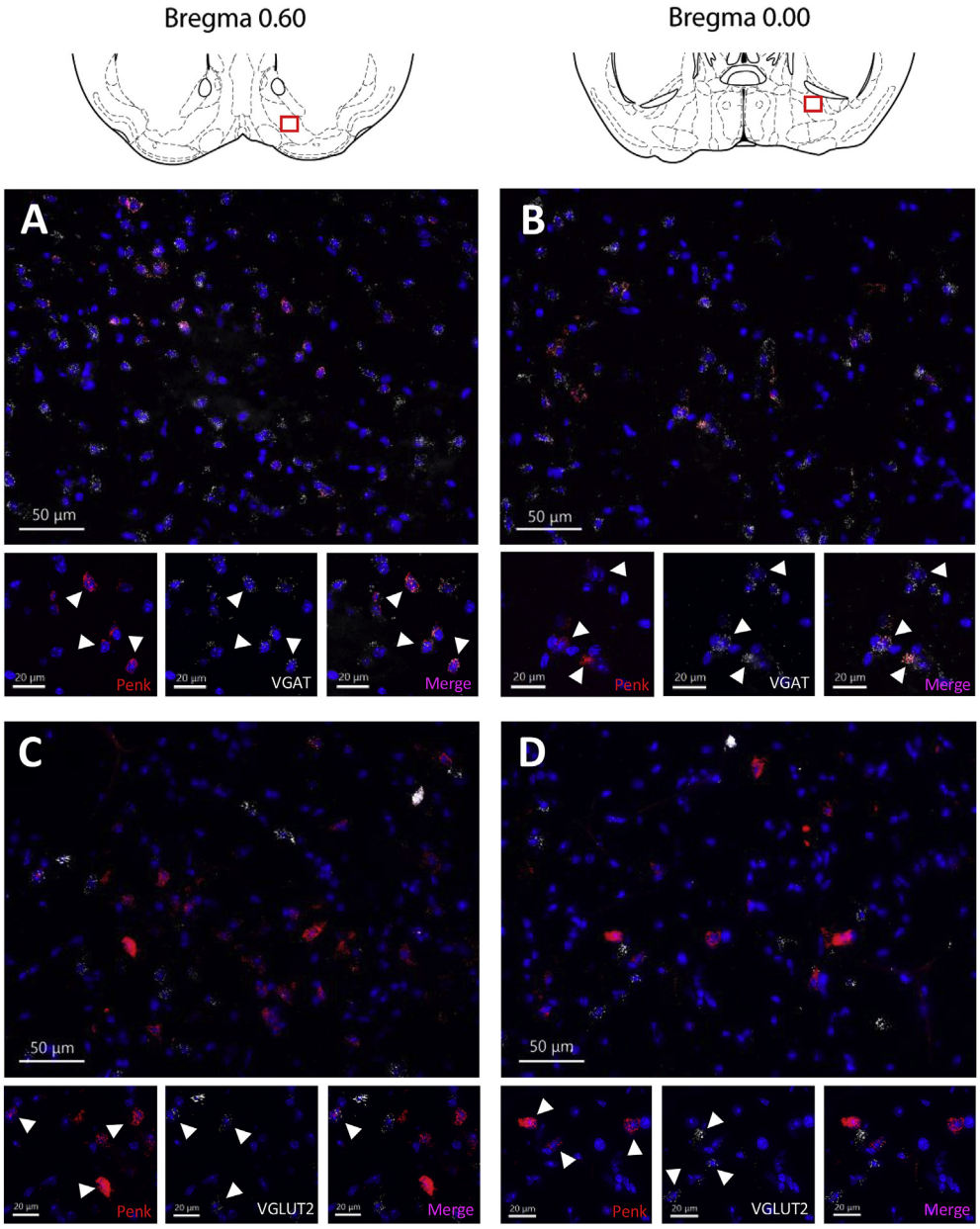

Fig. 1. Identification of neuron types within the ventral pallidum. Fluorescent *in situ* hybridization in wildtype mice (n = 3) to establish the expression pattern of preproenkephalin within rostral (**A&C**) and caudal (**B&D**) ventral pallidum (VP) slices (observed locations are indicated by red boxes in the histological diagrams above). Double labelling of preproenkephalin (Penk) (red) and either vesicular GABA transporter (VGAT) (**A&B**) or vesicular glutamate transporter 2 (VGLUT2) (**C&D**) (white) mRNAs. Penk was predominantly co-localized with VGAT in both the rostral and caudal VP and co-localized with VGLUT2 in either location. Insets under each image indicate examples of labeling of each separate probe as well as merged labelling. Arrows in insets below each image indicate neurons positive for the probe indicated, or neurons co-labeled in merged insets. The dark blue staining shows DAPI localization of cell nuclei.

VGLUT2 and 2.7% ± 0.8 (total: 11) showed co-labeling of Penk and VGLUT2 (Figs. 1C and 2B&D). In the caudal VP, 70.6% ± 3.5 (total: 371 of 525 DAPI-labeled neurons) were positively labeled for VGAT and 42.1% ± 4.1 (total: 221) were co-labeled for Penk and VGAT (Figs. 1B and 2H&I), while 16.8% ± 0.6 (total: 79 of 470 DAPI-labeled neurons) were positively labeled for VGLUT2 and 1.9% ± 0.9 (total: 9) were co-labeled for Penk and VGLUT2 (Figs. 1D and 2H&J).

Thus, in both the rostral and caudal VP, Penk was predominantly colocalized with VGAT-labeled neurons and rarely with VGLUT2-labeled neurons. Indeed, in the rostral VP, 95.1% ± 0.6 (total: 233) of 245 Penk-labeled neurons were co-labeled with VGAT and 63.1% ± 0.6 (total: 233) of 369 VGAT-labeled neurons were co-labeled with Penk (Figs. 1A and 2E), but just 5.9% ± 2.1 (total: 11) of 188 Penk-labeled neurons were co-labeled with VGLUT2 and 14.7% ± 5.1 (total: 11) of 75 VGLUT2-labeled neurons were co-labeled with Penk (Figs. 1C and 2F). Similarly, in the caudal VP, 92.9% ± 1.1 (total: 221) of 238 Penk-labeled neurons were co-labeled with VGAT and 59.6% ± 3.9 (total: 221) of 371 VGAT neurons were co-labeled with Penk (Figs. 1B and 2K), but just 4.9% ± 1.4 (total: 9) of 183 Penk-labeled neurons were co-labeled with VGLUT2 and 11.4% ± 7.9 (total: 9) of 79 VGLUT2-labeled neurons were co-labeled with Penk (Figs. 1D and 2L).

3.2. Confirmation of hM3Dq expression in preproenkephalin-expressing ventral pallidal neurons

To confirm the histological and functional expression of hM3Dq DREADD receptors in Penk-expressing VP neurons we performed FISH analysis of the VP in Penk-hM3Dq mice (n = 3) treated with CNO (0.1 mg/kg i.p. injection 2.5 h prior to perfusion) using probes for mCherry (tagged to hM3Dq receptors) and Fos. In the rostral VP, 60.4% ± 8.8 (total: 148) of 245 Penk-labeled neurons were found to be co-labeled with mCherry, and 98.7% ± 0.9 (total: 148) of 150 mCherry-labeled neurons were co-labeled with Penk (Supp Fig 2A). Similarly, 56.8% ± 4.1 (total: 130) of 229 Penk-labeled neurons were co-labeled with Fos, and 97.7% ± 0.1 (total: 130) of 133 Fos-labeled neurons were co-labeled with Penk (Supp Fig 2C). In the caudal VP, 55.0% ± 8.7 (total: 133) of 242 Penk-labeled neurons were found to

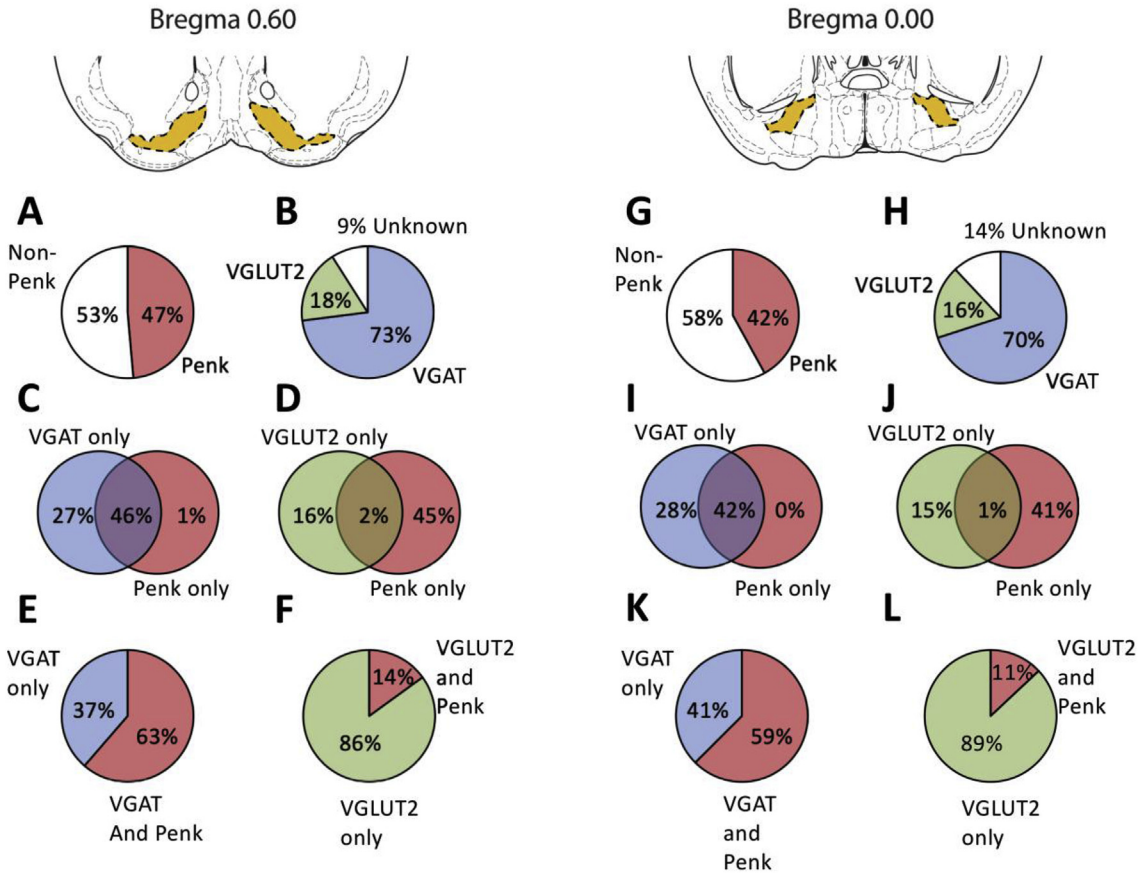

Fig. 2. Schematic of preproenkephalin, vesicular GABA transporter, and vesicular glutamate transporter 2 expression in ventral pallidum neurons. Fluorescent *in situ* hybridization analysis of the rostral (**A-F**) and caudal (**G-L**) ventral pallidum (VP) in wildtype mice (n = 3). Preproenkephalin (Penk)-labeled (**A&G**), vesicular GABA transporter (VGAT)-labeled (**B&H**), and vesicular glutamate transporter 2 (VGLUT2) (**B&H**) neurons, as well as Penk and VGAT (**C&I**) or VLUT2 (**D&J**) co-labeled neurons, as a percentage of the total number of identified neurons (DAPI-labeled). The percentage of VGAT (**E&K**) or VGLUT2 (**F&L**) neurons that were co-labeled with Penk. Histological images above indicate the rostral (left) and caudal (right) VP (in yellow).

be co-labeled with mCherry, and 98.5% ± 1.3 (total: 133) of 135 mCherry-labeled neurons were co-labeled with Penk (Supp Fig 2B). Similarly, 53.0% ± 4.8 (total: 123) of 232 Penk-labeled neurons were co-labeled with Fos, and 96.1% ± 0.9 (total: 123) of 128 Fos-labeled neurons were co-labeled with Penk (Supp Fig 2D).

These findings indicate that hM3Dq DREADD receptors were almost exclusively expressed in Penk-expressing VP neurons, and that CNO at the described dosage was sufficient to stimulate activity in Penk-expressing VP neurons via these expressed hM3Dq receptors.

3.3. Preproenkephalin-expressing ventral pallidal neurons do not control the acquisition of sign-tracking or goal-tracking in an autoshaping task

RNB inhibition of activity in Penk-expressing VP neurons did not alter the acquisition of sign-tracking responses in the autoshaping task, as indicated by a similar increase in CS + approaches and decrease in CS- approaches in both Penk-RNB (n = 8) and WT (n = 9) mice over six sessions (Fig. 3A; Significant CS*Session interaction ($F_{(5,75)} = 55.97$, $p < 0.001$), Non-significant CS*Session*Genotype interaction ($F_{(5,75)} = 0.71$, $p = 0.62$)). Similarly, the acquisition of goal-tracking responses did not significantly differ between Penk-RNB and WT mice (Fig. 3C; Significant main effect of Session ($F_{(5,75)} = 11.82$, $p < 0.001$), Non-significant CS*Session interaction ($F_{(5,75)} = 0.58$, $p = 0.72$), Non-significant CS*Session*Genotype interaction ($F_{(5,75)} = 0.20$, $p = 0.96$)).

DREADD activation of Penk-expressing VP neurons similarly had no effect on the acquisition of sign-tracking behavior in the autoshaping task. Indeed, both CNO- (n = 13) and saline-treated (n = 11) groups showed a similar increase in CS + approaches and decrease in CS- approaches over the course of the six conditioning sessions (Fig. 3B; Significant CS*Session interaction ($F_{(5,85)} = 38.99$, $p < 0.001$), Non-significant CS*Session*Genotype interaction ($F_{(5,85)} = 1.34$, $p = 0.26$)). Additionally, both CNO- and saline-treated groups showed an equivalent decrease in both CS+ and CS- goal-tracking responses (Fig. 3D; Significant main effect of Session ($F_{(5,85)} = 20.80$, $p < 0.001$), Non-significant CS*Session interaction ($F_{(5,85)} = 0.55$, $p = 0.74$), Non-significant CS*Session*Genotype interaction ($F_{(5,85)} = 0.15$, $p = 0.98$)).

These findings indicate that Penk-expressing VP neurons are not involved in the acquisition of sign-tracking or goal-tracking conditioned responses in the autoshaping task.

3.4. Activation of preproenkephalin-expressing ventral pallidal neurons impairs the acquisition of an inhibitory avoidance task

Following the completion of the autoshaping task, we tested the ability for aversive learning in Penk-RNB and Penk-hM3Dq mice using the one-trial inhibitory avoidance task. Prior to foot-shock inhibitory avoidance conditioning, no significant difference was observed between Penk-RNB (n = 8) and WT (n = 9) mice in the latency to enter the dark chamber (Fig. 4A; Non-significant main effect of Genotype ($F_{(1,15)} = 0.08$, $p = 0.78$)). Similarly, 24 h after conditioning, both groups showed a similar increase in the latency to enter the shock-paired (dark) chamber (Fig. 4A; Significant main effect of Conditioning ($F_{(1,15)} = 11.68$, $p < 0.01$), Non-significant Genotype*Conditioning interaction ($F_{(1,15)} = 0.25$, $p = 0.88$)).

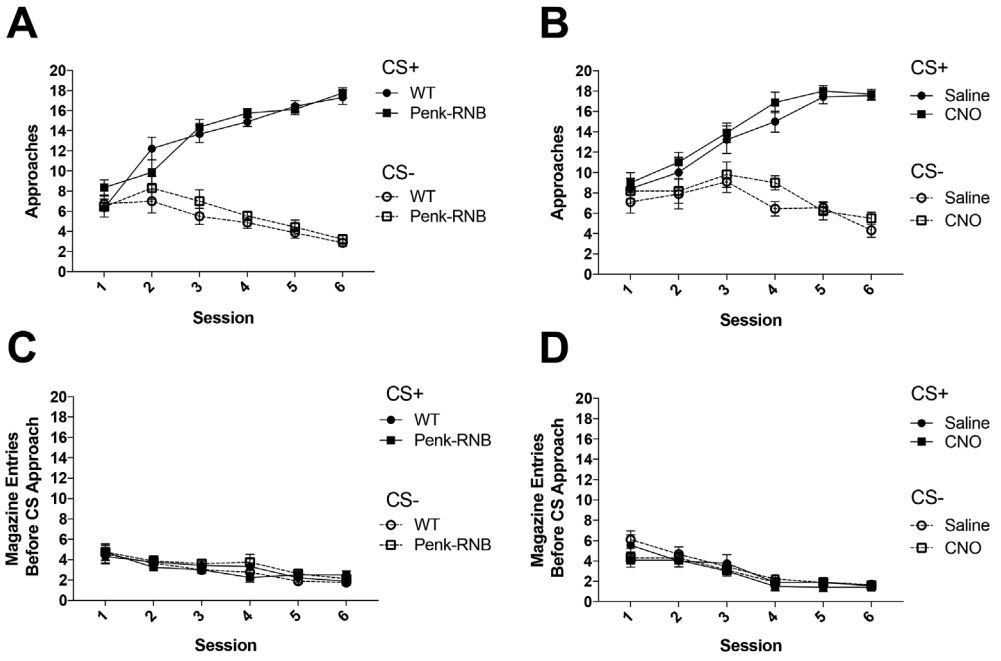

Fig. 3. DREADD excitation or RNB inhibition of ventral pallidum pre-proenkephalin-expressing neurons in an autoshaping task. (**A**) Sign-tracking and (**B**) goal-tracking behavior was not significantly altered by inhibition of Penk-expressing VP neurons in Penk-RNB (n = 8) mice compared with WT (n = 9) controls over six sessions. Similarly, in VP Penk-hM3Dq mice, excitation of VP Penk-expressing neurons by CNO (0.1 mg/kg) treatment (n = 13) did not significantly alter sign-tracking (**C**) and (**D**) goal-tracking behavior compared with controls treated with saline (n = 11). Values represent mean ± SEM.

In Penk-hM3Dq mice, CNO- (n = 13) and saline-treated (n = 11) mice did not differ in the latency to enter the dark chamber (Fig. 4B; Non-significant main effect of Treatment ($F_{(1,22)} = 1.26$, $p = 0.27$)). However, following avoidance conditioning, saline-treated, but not CNO-treated, mice showed an increased latency to enter the dark (shock-paired) chamber (Fig. 4B; Significant Treatment*Conditioning interaction ($F_{(1,22)} = 6.43$, $p < 0.01$), significant difference between saline and CNO groups post-conditioning in Student's t-test ($t = 2.37$, $p < 0.05$)).

These findings indicate that inhibition of Penk-expressing VP neurons is necessary for avoidance learning.

4. Discussion

Here, we showed that Penk-expressing VP neurons play an important role in the Pavlovian conditioning of environmental stimuli with aversive, but not appetitive, outcomes. Specifically, excitation, but not inhibition, of Penk-expressing VP neurons decreased the latency to enter a shock-paired chamber, indicating that inhibition of these neurons is necessary for inhibitory avoidance learning. These findings support previous studies highlighting the role of the VP in aversive learning in the inhibitory avoidance task (Lénárd et al., 2017; Péczely et al., 2014).

Previous evidence from our group revealed that activity in GABAergic NAc D2-MSNs, that would result in increased inhibition of the VP neurons to which they project, is necessary for aversive learning in the inhibitory avoidance task (Hikida et al., 2013, 2010). Indeed, decreased activity in NAc-projecting dopaminergic ventral tegmental (VTA) neurons, resulting in decreased NAc dopamine levels, increased the activity of D2-MSNs and facilitated inhibitory avoidance learning (Danjo et al., 2014). Thus, we suggest that during inhibitory avoidance learning, an aversive stimulus leads to a decrease the activity of VTA dopamine neurons, increasing the activity of NAc D2-MSNs, and resulting in inhibition of VP neurons. Indeed, the findings of the current study support this theory as inhibitory avoidance learning was able to occur in both wildtype and Penk-RNB mice, in which Penk-expressing VP neurons are inhibited by NAc D2-MSN inputs following an aversive stimulus or through RNB, respectively; however, DREADD stimulation of Penk-expressing VP neurons was able to disrupt the acquisition of inhibitory avoidance learning.

Our *in situ* hybridization examination of the VP revealed that Penk was predominantly expressed within GABAergic, rather than glutamatergic, neurons. Thus, decreased activity in these GABAergic Penk-expressing VP neurons would result in a disinhibition of their downstream targets. The mediodorsal thalamus, VTA, NAc, SNr, subthalamic nucleus, and the lateral habenula (LHb) are all known to be targets of VP

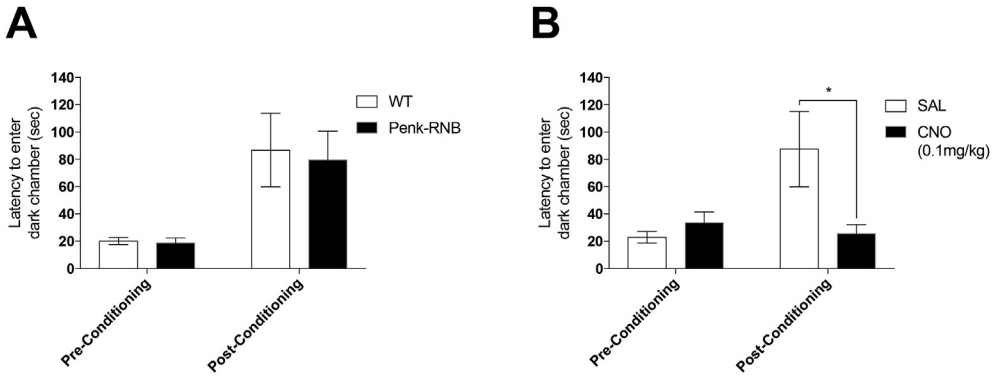

Fig. 4. Inhibitory avoidance learning following DREADD excitation or RNB inhibition of ventral pallidum pre-proenkephalin-expressing neurons. The latency to enter into a dark chamber from a light chamber was measured prior to aversive Pavlovian conditioning with an electric shock (Pre-Conditioning). Then, 24 h later (Post-Conditioning), the acquisition of inhibitory avoidance learning was measured by the latency to enter the shock-paired (dark) chamber. (**A**) Inhibition of ventral pallidum (VP) Penk-expressing neurons in Penk-RNB (n = 8) mice did not alter the acquisition of inhibitory avoidance learning compared with WT (n = 9) controls. (**B**) In VP Penk-hM3Dq mice, excitation of VP Penk-expressing neurons by CNO (0.1 mg/kg) treatment (n = 13) significantly reduced the latency to enter the shock-paired (dark) chamber compared with saline treatment (n = 11). Values represent mean ± SEM; student's t-test comparisons (*$p < 0.05$).

GABAergic efferent projections (Root et al., 2015). While it is still unclear which of these projection sites may be involved in inhibitory avoidance learning neurocircuitry, previous evidence has shown a significant role the LHb in controlling aversive learning. Activation of LHb neurons projecting to the VTA was sufficient to induce a conditioned place aversion (Friedman et al., 2011; Lammel et al., 2012), while inactivation of the LHb disrupted the long-term encoding of an aversive associative memory in the inhibitory avoidance task (Tomaiuolo et al., 2014). Thus, it is possible that aversive learning and its long-term storage may involve a VTA-NAc-VP-LHb-VTA loop.

Several studies have shown a role for the VP in behavioral responses to appetitive CSs, including cue-induced drug self-administration and cue-induced reinstatement of drug seeking (Mahler and Aston-Jones, 2012; Mahler et al., 2014). However, in the current study, excitation or inhibition of Penk-expressing neurons did not alter the acquisition of sign-tracking or goal-tracking conditioned responses. Our previous work has shown D1-MSNs in the NAc to control the attribution of incentive salience to the CS+, resulting in sign-tracking, in this task; however, our current findings suggest that these NAc D1-MSNs neurons likely do not project to Penk-expressing VP neurons (Macpherson and Hikida, 2018). It is still unclear whether VP neurons that do not express Penk, including a significant population of GABAergic neurons and the majority of glutamatergic neurons, are involved in reward learning in the autoshaping task or similar tasks involving appetitive associative learning, such as conditioned place preference.

In conclusion, here we demonstrate that inhibition of Penk-expressing VP neurons is necessary for inhibitory avoidance learning. Further study of the role of the VP in aversive associative learning neurocircuitry will likely be relevant for the identification of therapeutic targets for psychiatric conditions associated with aversive events, including post-traumatic stress disorder.

Competing interests

The authors declare that there are no conflicts of interest regarding this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.02.011.

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