Complex opioid driven modulation of glutamatergic and cholinergic neurotransmission in a GABAergic brain nucleus associated with emotion, reward and addiction.

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ABSTRACT

The medial habenula (mHb)/interpeduncular nucleus (IPN) circuitry is resident to divergent molecular, neurochemical and cellular components which, in concert, perform computations to drive emotion, reward and addiction behaviors. Although housing one of the most prominent mu opioid receptor (mOR) expression levels in the brain, remarkably little is known as to how they impact mHb/IPN circuit function at the granular level. In this study, our systematic functional and pharmacogenetic analyses demonstrate that mOR activation attenuates glutamatergic signaling whilst producing an opposing potentiation of glutamatergic/cholinergic co-transmission mediated by mHb substance P and cholinergic neurons, respectively. Intriguingly, this latter non-canonical augmentation is developmentally regulated only emerging during later postnatal stages. Further, specific potassium channels act as a molecular brake on nicotinic receptor signaling in the IPN with the opioid mediated potentiation of this arm of neurotransmission being operational only following attenuation of Kv1 function. Thus, mORs play a remarkably complex role in modulating the salience of distinct afferent inputs and transmitter modalities that ultimately influences synaptic recruitment of common downstream GABAergic IPN neurons. Together, these observations provide a framework for future investigations aimed at identifying the neural underpinnings of maladaptive behaviors that can emerge when endogenous or exogenous opioids, including potent synthetic analogs such as fentanyl, modulate or hijack this circuitry during the vulnerable stages of adolescence and in adulthood.

INTRODUCTION

Substance use disorders (SUDs) are agnostic to demographic group posing a widespread public health crisis impacting many communities[1]. Drugs of misuse influence synaptic function resulting in acute and protracted circuit adaptations that can exacerbate compulsive behaviors resulting in dependence[2,3]. Mu-opioid receptors (mORs) represent the primary molecular substrate responsible for both the clinical efficacious (i.e. pain management) and the euphoric effects promoting opioid misuse and addiction[4]. Additionally, mainly via liberation of endogenous opioids[5], mORs also facilitate the addictive propensity of other substances[5-7] e.g. alcohol and nicotine which, together with opioids, comprise the three most prevalent drugs of abuse worldwide[1]. mORs are expressed by varied neural cell-types resident in numerous regions within the brain[7-9]. Thus, detailed investigations as to how mOR activation impacts circuit dynamics constitutes an important endeavor critical for identification of potential therapeutic interventions aimed at alleviating the deleterious societal consequences of SUDs.

Here, we focus on a relatively understudied brain circuit comprising the medial habenula (mHb) and interpeduncular nucleus (IPN). The mHb is a bilateral epithalamic structure receiving significant synaptic input from, for example, the diagonal band and various septal regions [10-12]. The efferent output of the mHb is predominantly mediated by two distinct populations, substance P (SP) and cholinergic neurons, that primarily impinge on the IPN[11,13]. This latter structure in the ventral midbrain mainly houses GABAergic neurons that in turn influence activity in downstream brain regions including the raphe nuclei and the ventral tegmental area[14]. Thus, the mHb/IPN axis is anatomically positioned to integrate incoming limbic forebrain signals to ultimately modulate midbrain monoaminergic nuclei. It is therefore unsurprising that, perturbation/modulation of the synaptic recruitment or activity of IPN GABAergic neurons precipitates emotion, reward and addiction phenotypes[13-22].

The mHb/IPN axes houses one of the highest densities of mORs in the central nervous system[23,24] thus representing a prominent brain locus for opioid action. Despite this, descriptions regarding the role of mHb/IPN mORs in shaping behavior are still in their relative infancy[25,26]. mOR activation and subsequent G-protein signaling (Gi/o) classically produces an acute direct inhibitory effect in many neuronal types typified by membrane potential hyperpolarization via activation of inward rectifying potassium (GIRK) channels and/or reduced function of voltage-gated calcium channels function that are coupled to neurotransmitter release[8,27]. Strikingly, there is a dearth of information concerning the cellular effects and subsequent circuit influence upon mOR signaling in the mHb/IPN rendering such correlations speculative. Indeed, direct excitatory effects of mOR activation are apparent in certain areas of the nervous system either under

basal conditions or following chronic drug regimens[28-31]. Furthermore, within the IPN itself, $GABA_B$-receptor activation (another Gi-linked receptor) produces an increase in synaptic transmission[32-34] questioning whether this surprising effect is generalized to other members of this receptor family expressed within this region such as mORs.

The central role of the mHb/IPN circuitry underlying numerous behavioral aspects relating to emotion and the cycle of addiction, the conspicuous expression of mORs and a clear precedent for non-canonical effects of Gi-linked receptors together formed the impetus for the current work. Employing cell-type conditional optogenetics in combination with slice electrophysiology and pharmacogenetics we systematically dissect the effects of opioids on the mHb/IPN circuitry with a particular focus on synaptic signaling between these structures.

RESULTS

The two major neuronal populations of the mHb, substance P (SP) and cholinergic subtypes can be distinguished by virtue of *TAC1* and *CHAT* gene expression and largely segregate to the dorsal versus ventral mHb, respectively **(Fig. 1a,b)**. Together they provide much of the afferent input to the IPN via the fasciculus retroflexus (fr) axonal tracts **(Fig. 1b**) ultimately dictating IPN neuronal recruitment. The projection pattern as delineated by use of specific Cre transgenic mouse lines (ChATCre and Tac1Cre) when crossed to a conditional TdTomato reporter (Ai9), reveals a predominant innervation of IPL by SP neurons whereas cholinergic neurons impinge on the IPR/IPC subdvisions (**Fig. 1b**). As previously reported[23], we confirm here that mOR protein expression is notable in the mHb, the fasciculus retroflexus axonal tracts and particularly prevalent in the IPN **(Fig. 1c)**. Within this latter structure, high resolution microscopy clearly show mOR expression is densest in the lateral IPN (IPL) with intermediate and relatively lower levels found in the rostral IPN (IPR) and central IPN (IPC) subregions, respectively (**Fig. 1c**). Selective stimulation of each of these mHb neuronal populations in isolation is achieved using Cre-mediated conditional expression of channel rhodopsin by crossing Ai32 mice with either ChATCre or TAC1Cre mice; see methods for details). Adopting this optogenetic approach we investigate how mOR receptor activation modulates habenulo-interpeduncular synaptic dialogue imparted by these parallel yet distinct afferent systems in adult mice (p>40). Both SP and cholinergic neurons express the glutamate vesicular transporter, VGlut1, and hence competently release this excitatory neurotransmitter[11,21,35].

mOR activation reduces substance P neuronal mediated glutamatergic transmission onto IPL GABAergic neurons

Since the highest expression of mOR was found in the IPL, we initially probed the effect of a selective mOR agonist (DAMGO, 500 nM) on AMPA receptor mediated transmission mediated by SP neurons that innervate this subregion. DAMGO application results in a significant decrease in light-driven (470 nM; typically, 10-50 % arbitrary LED power equating to approximately 0.4 – 3.4 mW/mm^2; CoolLED illumination system) AMPAR mediated EPSCs in TAC1Cre:Ai32 mice that is partially reversed upon washout of the agonist(**Fig. 1d,e**). This is accompanied by an increase in S2/S1 paired pulse ratio (PPR) suggesting the modulation is driven by presynaptic changes in release probability(**Fig. 1d,f**). Thus, neurotransmitter release by this specific afferent input to the IPL is directly, negatively modulated like that seen in many other neural circuits within the brain[8].

mOR activation augments the strength and fidelity of glutamatergic transmission by cholinergic neurons resulting in enhanced excitation-spike coupling in IPR GABAergic neurons

We next examined how mORs modulate mHb-IPN neurotransmission that occurs via the other major input mediated by cholinergic neurons. To this end, we employed ChATCre:ai32 mice and focused on the IPR subdivision (**Fig. 2a**) due to the relatively high levels of combined mOR expression and cholinergic neuronal innervation (**Fig. 1b,c**) in this subregion. Remarkably, DAMGO application elicited a significant robust and reversible potentiation of light-evoked (470 nM; typically, 10-100 % arbitrary LED power equating to approximately 0.4 – 6.9 mW/mm^2; CoolLED illumination system) AMPAR EPSC amplitude (**Fig. 2a,b**) in stark contrast to that seen with mHb TAC1-IPL glutamatergic signaling described previously (**Fig. 1d,e versus Fig. 2a,b**). Notably, the accompanying PPR changes were not consistent with an increase in release probability raising uncertainty as to the synaptic locus of the mOR effect (**Fig. 2c**). We considered non-specific actions of DAMGO such as those mediated by direct modulation of ChR2 itself to explain this non-canonical observation. However, our previous data demonstrating the reduction of glutamatergic neurotransmission mediated by SP neurons by DAMGO described (**Fig. 1**) renders this possibility unlikely. Furthermore, a similar potentiation of cholinergic neuronal mediated glutamatergic transmission in the IPN upon activation of $GABA_B$ receptors has demonstrated a propensity for this circuit to undergo such modulation in response to a Gi-linked receptor[32-34]. Nevertheless, despite this precedent, we employed a pharmacological approach to further validate this unexpected result. We show that lower

concentrations of DAMGO (100 nM) and alternative mOR agonists, Met-enkephalin (Met-enk, 3 μM) and morphine (10 μM) all induce potentiation of ChAT neuronal glutamatergic transmission to similar extents (**Fig. 2d**). In addition, pre-treatment with the mOR selective antagonist (CTAP, 1mM) completely prevents the DAMGO induced response (**Fig. 2d**). Together, this pharmacological battery of tests employing an experimental, an endogenous and clinical/recreational mOR agonists and selective mOR antagonism clearly implicate this opioid receptor in mediating a non-canonical potentiation of AMPAR EPSC amplitude elicited by mHb cholinergic neurons in IPN.

In several recordings, no measurable post synaptic AMPA receptor EPSC could be elicited in IPR neurons following light-evoked stimulation of cholinergic axons even with the maximal possible light intensity available (i.e. 100 % arbitrary LED power; approximately 6.9 mW/mm^2), and thus further interrogation was typically not performed. However, in a few instances, we nevertheless proceeded with DAMGO or Met-enk application and surprisingly in 7/11 recorded IPR neurons a robust and reversible emergence of a significant AMPAR mediated EPSC was observed (**Fig. 2e,f**). Thus, these data clearly reveal that mOR activation invokes a synaptic mechanism that, at its most extreme, results in an “un-silencing” of ChAT neuronal glutamatergic transmission in IPN. Since, PPR measurements are only valid if the same population of release sites are assayed before and after experimental manipulation, the additional engagement of a putative reluctant vesicular pool[32] by mOR activation could explain the discrepancy between the augmentation of EPSC amplitude and observed changes in PPR (**Fig. 2b,c**). Future interrogation is required to definitively identify the relative contribution of pre- and post-synaptic loci to the potentiation observed.

To date, we have examined the effect of mOR on synaptic transmission during paired pulse light activation delivered at 20 Hz. Although, mHb cholinergic neurons are capable of burst firing at much higher frequencies in response to afferent stimulation *in vivo*[12], they typically exhibit low frequency (i.e <10Hz) intrinsic (i.e. independent of synaptic input) firing *in vitro*[10,36-38]. In agreement with these previous studies, cholinergic neurons in ventral mHb (identified in ChATCre:Ai9 mice) spontaneously elicit action potentials measured in cell-attached recordings at an average of ~5 Hz (range 2 - 10Hz; **Fig. 3a**). Therefore, we assessed the role of mOR activation on glutamatergic transmission mediated by cholinergic neurons elicited by light stimulation trains delivered at this frequency. Interestingly, even with this relatively low frequency paradigm, glutamatergic transmission is extremely labile in nature as evidenced by the large and rapid depression of AMPAR EPSC during the stimulus train (**Fig. 3b,c**). Remarkably, activation of mORs essentially eliminates activity-dependent depression at this synapse. This

switch in transmission dynamics greatly facilitates the probability of excitatory postsynaptic potential-spike coupling (ES coupling) in response to each stimulus within the train (**Fig. 3d-e**). Thus, these data clearly demonstrate that at physiologically relevant stimulus patterns, mORs serve to dramatically increase the fidelity of glutamatergic transmission in the IPR mediated by cholinergic neurons culminating in an enhanced recruitment of postsynaptic IPR neurons.

mORs constitutes a molecular switch to alter the salience of glutamatergic transmission mediated by cholinergic and substance P neurons in the IPR

An intriguing inconsistency was observed regarding the DAMGO effect on light-evoked and spontaneous EPSCs (sEPSCs) impinging on IPR neurons. Under the experimental conditions employed, as observed with light evoked EPSCs, sEPSC events were exclusively mediated by AMPARs as evidenced by a virtually complete cessation upon DNQX application (**Fig. 4a**). Remarkably, in contrast to the previously described robust potentiation of light-evoked AMPAR EPSCs, activation of mORs significantly attenuates sEPSC frequency with no effect on amplitude (**Fig. 4b-d).** Thus, in a single IPR postsynaptic neuron opposing effects on light-evoked and spontaneous AMPAR EPSCs is evident (**Fig. 4e**). Prevailing circuit schemas based on axonal arborization patterns portray a mutually exclusive, non-overlapping afferent input to IPN mediated by cholinergic and SP neurons to IPR/IPC versus IPL, respectively. However, in the current study by employing high-resolution imaging in Tac1Cre:Ai32 mice a clear presence of synaptic bouton-like structures in the IPR is noted. These structures do not co-localize with endogenous ChAT, but do express the glutamate vesicular transporter, VGluT1 (**Fig. 4g,h**). This presence of putative presynaptic anatomical substrates points to the existence of a secondary input to IPR distinct to the well-established one originating from mHb cholinergic neurons. Indeed, significant light-evoked AMPAR EPSCs in IPR neurons can be elicited in Tac1Cre:Ai32 mice with essentially similar amplitudes across all LED powers employed to that seen in ChATCre:Ai32 mice (**Fig. 4i**). Thus, in contrast to recent ultrastructural EM analyses[32], these data indicate a previously unidentified glutamatergic input to IPR mediated by SP neurons. Interestingly, DAMGO application significantly depresses the SP neuronal glutamatergic output in IPR and increases PPR (**Fig. 5a-c**) to a similar extent to that observed in IPL (**Fig. 1d,e; Fig. 7i**). This is in direct contrast to the role of mORs in positively modulating the ChAT neuronal glutamatergic transmission in the IPR (**Fig. 2a,b; Fig. 7i**). Thus, for the first time we demonstrate both SP and cholinergic input to the same subregion of IPN and that mOR activation elicits diametrically opposing effect on transmission mediated by these respective presynaptic neuronal populations.

Dynamic regulation of the mOR elicited afferent specific switch in salience of the glutamatergic transmission to the IPR during adolescence

Our functional studies thus far have been restricted to sexually mature, adult mice (> p40). mOR expression emerges in many brain structures during prenatal development largely overlapping with their final adult distribution profile by mid/late gestation[39]. In agreement, we reveal that mOR protein expression in IPR is present at appreciable levels from early postnatal stages (from P5 onwards; **Fig. 5d**). This begs the question whether modulation of interpeduncular synaptic transmission described during these critical early epochs mirrors that seen in the adult. To examine this, we extended our analyses to earlier time points (P15 to P40) and found that mOR modulation of glutamatergic transmission mediated by cholinergic neurons in the IPR undergoes a remarkable developmental regulation. This is characterized by an initial inhibitory effect on light-evoked AMPAR EPSCs transitioning to the previously described augmentation(**Fig. 5e,f,i**). In contrast, the glutamatergic input to the IPL and the newly discovered input to the IPR elicited by SP neurons (**Fig. 4i**) is not developmentally regulated with mOR activation being consistently inhibited by DAMGO application at all ages tested (**Fig. 5g-i**). Thus, during early development opioids result in overall inhibition of afferent input to the IPR agnostic to afferent input with the emergence of the previously highlighted differential regulation of mHB cholinergic versus substance P neuronal output emerging during the late adolescent stages (**Fig. 4i).**

mOR induced potentiation of nicotinic receptor signaling in IPR is conditional on the removal of a molecular brake mediated by Kv1 channel function

In addition to mORs, this circuitry contains dense expression of nicotinic receptors located at pre- and postsynaptic sites[10,40,41]. However, the ability to reliably evoke post-synaptic nicotinic receptor (nAchR) EPSCs in response to physiologically appropriate stimuli has been challenging. Indeed, many studies have resorted to high frequency and prolonged stimulation[42] or bath/puff applications of nicotine to investigate nAChR function in this circuit[19,36,43-46]. Despite the faithful co-expression of endogenous ChAT in ChatCre:Ai32 expressing terminals located in the IPR(**Fig. 6a**), in agreement with previous observations[42,46], evoked transmission by brief light pulses (1-5ms) from cholinergic neurons predominantly elicits pure AMPAR EPSC in postsynaptic IPR neurons under the experimental conditions employed (**Fig. 4a; Supplementary Fig. 1a-c**). Additionally, DAMGO, at concentrations that strongly potentiate glutamatergic transmission, does not result in a measurable postsynaptic nAChR EPSC after AMPARs are pharmacologically silenced. (**Supplementary Fig. 1a-c**). Conversely, the

potentiated ESPC following mOR activation is purely AMPAR mediated as evidence by complete block by DNQX (**Supplementary Fig. 1d-f**). Thus, brief pulses of light (1-5ms) in ChatCre:ai32 mice does not result in sufficient ACh release, if any, to produce measurable postsynaptic nAChR response even under conditions where mOR activation strongly enhances glutamatergic transmission at these synapses. Interestingly, cholinergic transmission at the neuromuscular junction (NMJ) can be boosted via potassium channel blockade with fampridine (4-aminopyridine) and has been clinically indicated in disorders such as myasthenia gravis and multiple sclerosis[47]. Furthermore, low micromolar 4-AP in slices amplifies acetylcholine release, as assessed by Grab-ACh mediated fluorescence in the IPN[48,49]. Experimentally, these concentrations are relatively selective in inhibiting K^+-channels of the shaker family (Kv1). Interestingly, of the members of this family, *KCNA2* (the gene encoding Kv1.2) mRNA is highly expressed in ventral habenula (**Fig. 6b**) a region that is enriched in cholinergic neurons (**Fig. 1a**) . We therefore speculated that Kv1.2 block could result in enhancement of ACh release to perhaps reveal postsynaptic nAChR EPSCs in IPR neurons. Indeed, following block of glutamatergic transmission (DNQX + APV), subsequent application of 4-AP (50-100 μM) or the more selective Kv1 channel antagonist, dendrotoxin-α (100 nM) results in the emergence of an EPSC elicited by brief light pulses including in response to trains of 5Hz stimulation (**Fig. 6c,d**). This emergent EPSC is mediated by nAChRs as evidenced by ~ 80-90% block by the nicotinic receptor antagonist, DHβE[50,51] (2 μM; **Fig. 6d,e**). Interestingly, this approach allows for the calculation of nAChR;AMPAR peak amplitude ratio as a measure of the relative contributions of these components of co-transmission within an individual postsynaptic IPR neuron (**Fig. 6g**). As an alternative approach we also employed the ChAT-ChR2 mouse. nAChR EPSCs could also be elicited in this mouse line following 4-AP application, although the measured nAChR:AMPAR ratio is higher than that seen in ChatCre:ai32 mice (**Fig. 6g**) indicating a skew towards more prominent cholinergic transmission when compared to that mediated by glutamate. It is unclear as to the exact reason for this divergence, but it may be related to increased levels of the vesicular Ach transporter (vAChT) expression observed in ChAT:ChR2 mice[52]. In Tac1Cre:Ai32 mice, 4-AP application following block of glutamatergic transmission does not result in emergence of any EPSC thus nAChR:AMPAR ratios is essentially 0 (**Fig. 6g**) Finally, application of a cholinesterase inhibitor (ambenonium, 50 nM) markedly prolongs the EPSC waveform with minimal effect on amplitude (**Fig. 6h**). Thus together, these results corroborate the role of Kv1.2 as a molecular brake, removal of which, results in the emergence of habenulo-interpeduncular synaptic transmission via postsynaptic nAChRs on IPR GABAergic neurons.

Having established the ability to reliably assess an otherwise reluctant cholinergic transmission, we subsequently investigate how nAChR mediated signaling in the IPR is impacted by mOR activation. As with AMPAR mediated EPSCs, DAMGO produces a marked potentiation of light-evoked nAChR EPSC amplitude in IPR that is completely blocked by DHβE (**Fig. 7a,b**). The mOR-mediated potentiation of both the glutamatergic and cholinergic output is essentially similar (**Fig. 7i**) Till now, we have utilized optogenetic approaches to assay synaptic transmission in the IPN. Interestingly, following acute block of Kv1, nAChR EPSCs could also be elicited via electrical stimulation (**Fig. 7c**). Further, DAMGO reliably potentiated both electrical and light evoked nAChR EPSCs in a single IPR neuron to similar extents, a response that is blocked by DHβE (**Fig. 7c-e**). Finally, we tested the molecular specificity of the DAMGO response employing Chat-ChR2:global *OPRM1* knockout mice. In these mice DAMGO application is ineffective in augmenting glutamatergic or cholinergic mediated neurotransmission in IPR whilst the potentiation upon $GABA_B$ receptor activation previously described[32,34,53] remains intact (**Fig. 7f-h;** note that in these experiments CGP55845A which is routinely included in all other experiments was omitted). This genetic approach complements our previous pharmacological data (**Fig. 2d**) to directly implicate mORs in the potentiation of mHB-IPN transmission. The overall effects of mOR activation on synaptic transmission mediated by the distinct mHb afferent systems in the various subdivisions of IPN tested is summarized in **Fig. 7i**.

DISCUSSION

In the current study, we reveal for the first time a remarkable augmentation of glutamatergic/cholinergic co-transmission by mHb cholinergic neurons following mOR activation in the IPN. However, the underlying cellular/network mechanisms responsible remain unclear. The most parsimonious explanation is that mORs in proximity to the release machinery of cholinergic nerve terminals[23] mediate this effect. However, one must consolidate the fact that a Gi-linked receptor, which typically serve to directly inhibit release, results in a seemingly paradoxical potentiation. Functional and mechanistic studies have elegantly demonstrated that $GABA_B$ receptors (another member of the Gi-linked subfamily) trigger acute molecular and structural adaptations resulting in enhanced Ca^{2+} influx and a switch of transmission modes within single presynaptic terminals to ultimately increase neurotransmitter release[32,33]. Although, these observations set a precedent for potentiation by Gi-linked receptor activation in the IPN similar experimental approaches to that employed in the examination of the $GABA_B$-receptor mediated potentiation[32,33,49] are required to definitively implicate overlapping mechanisms. In

other brain regions, a canonical inhibitory influence of mORs elicits overall network excitation via disinhibition[54-57]. Furthermore, numerous studies have revealed an intricate interplay involving diverse neuromodulatory components intrinsic to the IPN that serve to regulate synaptic transmission[15,53,58]. Thus, at present we cannot discount the possibility that the potentiation observed here may result via the activation of mORs residing on other neural elements within the IPN microcircuitry such as postsynaptic neurons or glia, for instance. Regardless, of the exact underlying cellular and/or network mechanisms our data extend the previously described Gi-linked receptor potentiation of neurotransmission in the IPN[32,33,49] to mORs, a predominant target of a societally relevant and prevalently misused class of drugs.

Recent generation of transgenic mice[24,59,60] have permitted manipulation of mOR-expressing neurons and mOR receptors including those expressed specifically within the mHb/IPN. Emerging behavioral studies adopting such conditional genetic approaches have highlighted an important role of mORs within this circuitry in mediating varying aspects of reward and aversion[25,26]. Unsurprisingly, it has been concluded that the observed behavioral effects of such manipulations are due to perturbations of a generalized inhibition mediated by mORs within the mHb/IPN circuit. Here, we reveal a functional dichotomy characterized by an inhibitory yet excitatory influence on neurotransmission mediated by SP and cholinergic neurons, respectively. These distinct neuronal populations participate in and drive specific behavioral facets demonstrating a division of labor. For example, SP neuronal activity positively correlates with reward outcome, history and hedonic value[61,62], whereas that of cholinergic neurons is reduced during behaviors associated with reward[61]. mHb cholinergic neurons have also been linked to negative affect including anxiety and depression[38,63,64] that promote drug-seeking behaviors during withdrawal[65]. We reveal for the first time that the IPR subdivision receives functional glutamatergic afferent input from SP neurons that based on previous anatomical interrogation were considered to exclusively target the IPL[66]. Interestingly, the novel opposing effect on transmission extends to the IPR thus demonstrating a role for mORs in altering the relative salience of these distinct afferent systems with regard recruitment of common downstream IPR neurons. The IPR houses somatostatin (SST) GABAergic neurons whose afferents impinge on the raphe nucleus and lateral dorsal tegmental nucleus, the latter in turn influencing the VTA and its downstream structures[15,46]. Thus, careful consideration regarding future examination of behavioral outcomes resulting from the described contrasting effects of mOR activation on the parallel processing of reward (mHB SP) and anti-reward (mHB ChAT) in this circuit is essential.

Throughout this study we contextualize the effect of mOR activation in the IPN primarily through the lens of exogenously introduced opioids. However, physiological activation of mORs can be mediated to differing extents by endogenous ligands such as dynorphin or enkephalin. Indeed, we demonstrate that met-enkephalin effectively boosts co-transmission from cholinergic neurons in a similar manner to morphine. Interestingly the IPN is home to a population of pro-enkephalin (PENK)-expressing neurons and detectable met-enkephalin immunoreactivity[23,67]. In other brain regions, manipulation of PENK neuronal activity and or ablation of PENK itself elicits mOR mediated circuit modulation and behavioral alterations[68-70]. It is anticipated that activation of mORs by local endogenous opioid release yields similar complex effects to those following exogenous application of mOR agonists described here. Thus, this physiological route will have major implications concerning the role of mHB/IPN mORs in mediating the positive reinforcing effects of non-opioid drugs of abuse (e.g. nicotine, alcohol, amphetamines) and other “natural” rewarding stimuli (e.g. such as those associated with social interaction, exercise and food intake) thus extending the relevance of our study to these additional modalities.

Another novel finding in the current study relates to the identification of a “molecular brake” that exerts strong control over nAChR signaling impinging on postsynaptic IPN neurons. Specifically, compromising the function of delayed rectifying K^+-channels containing Kv1.2 subunits results in emergence of nAChR EPSCs in response to light and electrical stimuli delivered with physiological paradigms likely thru increased axonal/bouton excitability and calcium influx via action potential waveform modulation. Kv1.2 function can be bidirectionally impacted by use-dependent mechanisms or through secondary activity mediated cascades resulting in post-translational modifications (e.g. phosphorylation state) that impact its function and/or trafficking[71-73]. These plausible endogenous mechanistic routes could serve to titer the strength of transmission via nAChRs in the IPR. The resident nAChRs in the mHb/IPN are primarily encoded by the CHRNA3/B4/A5 gene cluster and the role of this circuitry in nicotine use is well characterized. For example, increased propensity for nicotine abuse in humans is associated with dysregulation of nAChR function precipitated by single nucleotide polymorphisms of this cluster[74-76] and experimental manipulation of nAChR signaling in the mHb/IPN generates phenotypes associated with various aspects of nicotine consumption in mouse models[51,77-80]. Furthermore, chronic nicotine results in adaptations in nAChR expression and signaling that can exacerbate continued nicotine consumption[36,45,81,82]. Together, these studies establish a link between nAChR mediated signaling within this circuitry and prevalence of nicotine misuse. Here we highlight an intriguing interplay between opioid and cholinergic systems within the mHb/IPN axes. Our data clearly demonstrate that mORs and Kv1.2 together

comprise synergistic molecular targets that, in addition to others previously identified[34], when leveraged in tandem can manipulate nAChR mediated signaling to yield potential interventions for nicotine overuse.

Adolescence is a critical period for many aspects of brain and social development and represents a high-risk demographic group for drug use developing into long-term addiction[83]. Propelled by the societal introduction of highly potent synthetic morphine analogs (e.g fentanyl), one of the many devastating sequalae of the well-documented opioid crisis is an alarming high rate of overdose deaths not only in adults but also during vulnerable teenage years. Of note is the striking valence conversion from depression to potentiation of mHb ChAT neuronal signaling in the IPR occurring around the late postnatal stages. This is in stark contrast to that seen with the newly described SP neuronal mediated transmission in this same IPN subdivision where mOR activation is inhibitory at all life stages assayed. Thus, during postnatal development opioids acting via mORs result in a blanket inhibition of these two distinct inputs prior to the establishment of the afferent specific modulation in the adult. Interestingly, numerous studies have highlighted changes in the role of mORs in reward associated behaviors coinciding with similar periods[84,85]. An attractive hypothesis is that coordinated modifications at the cellular, molecular and/or network level underlie this dynamic developmental regulation. Thus, our circuit analyses provide insights into the neural correlates for the differing propensity of mORs to regulate not only positive reinforcement but also the negative effects associated with withdrawal at various stages of development[86]. Whether these adaptations underlie the increased propensity for substance abuse during development remains to be ascertained. Furthermore, opioid exposure during early developmental epochs imparts long lasting effects on the brains reward circuitry[3]. Thus together, it is imperative to consider the newly described evolving role of mORs in the mHb/IPN circuitry. This is particularly relevant with respect to generation of potential preventative treatments for the deleterious consequences of fetal opioid exposure and SUDs in high-risk juveniles.

In addition to a central role in addiction, the mHb/IPN circuitry encodes fear associated behaviors[32,33,87-89]. Interestingly, prevention of $GABA_B$-receptor function in mHb cholinergic neurons or increasing IPN neuronal activity facilitates and augments expression of fear extinction, respectively[32,33], Dysregulation of this behavioral aspect is a key underlying cause of post-traumatic stress disorder (PTSD)[90]. Based on this previous work, the novel mOR mediated potentiation of the mHb cholinergic transmission and hence IPN neuronal recruitment described here constitutes a parallel, yet alternative circuit mechanism involved in the extinction of learnt fear. Thus, pharmacogenetic manipulation of mOR mediated signaling specifically in the IPN

may constitute a viable approach to alleviate conditions associated with abnormal fear processing such as those seen in PTSD.

In summary, we highlight several previously undescribed and hence unappreciated roles of mORs in the dynamic regulation of synaptic transmission impinging on IPN GABAergic neurons following exposure to exogenous opioids. Together these detailed functional analyses have yielded important findings that necessitate consideration in future interrogation concerning the role of these receptors in modulating a critical, yet relatively understudied, brain circuitry which encodes varying aspects of emotion, hedonic and addiction behaviors.

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METHODS

Animals

All experiments were conducted under an active animal protocol approved by the Animal Care Use Committee of the National Institute of Child Health and Human Development. All transgenic mice were attained from The Jackson Laboratory (ME, USA) and were as follows: ChatCre (), Tac1Cre(), Ai9(), Ai32(), CHAT-ChR2(); OPRM1 KO (). For conditional expression of td-Tomato (AI9) or ChR2 (Ai32) both male and female breeders were homozygous, and all immunocytochemical or electrophysiological experiments were performed in their progeny that were heterozygous for both the Cre and floxed alleles. Heterozygous ChAT-ChR2 mice were used throughout the study either alone or crossed with the OPRM1KO mouse (homozygous). Both male and female mice were investigated, and the data were pooled.

Reagents

Pharmacological reagents used in this study are as follows: DNQX (Cat. No. 2312/10), DL-APV (Cat. No 0105/10), CGP55845A (Cat. No. 1248/10), Picrotoxin (Cat. No. 1128), bicuculiine (Cat. No. 0109/10)**,** DAMGO (Cat. No. 1171/1), CTAP (Cat. No. 1560/1) and DHβE (Cat. No. 2349) were all purchased from Biotechne/Tocris (MN, USA); Dendroroxin-α (Cat No. D-350) was purchased from Alomone Labs (Jerusalem, Israel). met-enkephalin (Cat. No. M6638**)** was purchased from MilliporeSigma (MA, USA). Ambenonium (Cat. No. sc-203507) was purchased from Santa Cruz Biotechnology (TX, USA) Morphine Sulphate (Cat. No. NDC: 0641-6125) was from Hikma (NJ, USA) and attained via the NIH Division of Veterinary Resources (DVR) Pharmacy.

Primary and corresponding secondary antibodies used in this study for immunocytochemistry are as follows: Guinea pig anti-RFP (Synaptic Systems, Cat. No. 390005, working concentration: 1ug/ml) with CF555-conjugated Donkey anti-Guinea Pig IgG (Biotium Cat. No. 20276, 1:1000 working dilution).Chicken anti-GFP (AvesLabs, Cat.No. GFP-1010, working concentration: 2.5ug/ml) with CF488-conjugated Donkey anti-Chicken IgY (Biotium Cat. No. 20166, 1:1000 working dilution). Rabbit anti-MOR (ABCAM, Cat. No. ab134054, working dilution: 1:500) with CF555-conjugated Donkey anti-Rabbit IgG (Biotium cant#20038, 1:1000 working dilution). Goat anti-Chat, Millipore (Cat. No. AB144P, working dilution: 1:1000) with CF555-conjugated Donkey anti-Goat IgG (Biotium, Cat. No. 20039, 1:1000 working dilution).

Immunocytochemistry and Imaging

Mice (P>15) were perfused trans-cardinally using 4% PFA and dissected brain tissues were post-fixed in 4% PFA for 24 hours at 4 °C. For P5 mice, brains were removed and drop fixed in 4% PFA for 24 hours at 4 °C. Fixed brain tissues were thoroughly washed in 1x phosphate

buffer (PB) followed by cryopreservation using 30% sucrose. 50 µm coronal sections were made on a frozen microtome. To perform floating section IHC, brain slices were washed with 1x PB at room temperature for 1 hour with 2-3 changes of 1x PB followed by blocking and permeabilizing in Blocking Solution (1x PB + 10% donkey serum + 0.5% Triton X-100) at room temperature for at least 2 hours. Blocked brain slices were incubated in primary antibodies, which were diluted to working concentration using Antibody Solution (1x PB + 1% donkey serum + 0.1% Triton X-100), at 4 °C for 24-48 hours. After wash with 1x PB at room temperature for 15 minutes with 3 repeats, brain slices were incubated in secondary antibodies diluted with Antibody Solution at room temperature for 1 hour, followed by DAPI staining at 1ug/ml for additional 15 minutes. After washed with 1x PB at room temperature for 15 minutes with 3 repeats, brain slices were mounted on gelatin coated slides followed by air drying, covered with No.1.5 cover slides ProLong Glass Antifade Mountant (ThermoFisher Scientific, Cat#P36984), cured in darkness overnight before imaging. Confocal images were acquired on a Zeiss LSM900 using ZenBlue software. Light path and scan settings were conFig.d for best signal with multiplex channels. 10x and 20x confocal images of whole habenula and IPN were acquired as tiles with z-steps of 3µmx5 and 0.9µmx20, respectively. Airy scan images were acquired at 63x using multiplex 2Y mode to achieve super resolution. 2µm depth of images were captured at z-steps of 0.2µmx10, with or without tiling. All comparable images of tissues with different genotypes or developmental stages were acquired using the same light path configuration, channel input, gain and offset. Images were imported into Fiji and Adobe Photoshop for processing.

Electrophysiology

P15-P91 mice (for details refer to the results section) were anesthetized with isoflurane, and the brain removed in ice–cold partial sucrose substituted ASCF (ssACSF) solution containing (in mM): 90 Sucrose, 80 NaCl, 25 NaHCO3, 1.25 NaH2PO4, 3.5 KCl, 4.5 MgCl2, 0.5 CaCl2, 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4; osmolarity 300-310 mOsm). Coronal sections containing wither the mHB or IPN (270 - 300 µm) were cut using a VT–1000S vibratome (Leica Microsystems, Bannockburn, IL) in ice-cold ssACSF. The slices were allowed to recover in the ssACSF at 31-33°C for 20-30 minutes followed by an additional 20 minutes at room temperature. Slices were then transferred to our standard extracellular solution (ACSF) of the following composition (in mM): 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$, and 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4; osmolarity 300-310 mOsm) for storage until electrophysiological recording. Slices were transferred to an upright

Olympus BX51WI microscope and visualized with infrared differential interference contrast microscopy and perfused (2-4 ml/min) with the above extracellular solution. Unless otherwise indicated the recording extracellular solution was routinely supplemented with 2μM CGP55845A, 50μM picrotoxin and 10 μM bicuculline (Tocris Science or Ascent Scientific).

All recordings were performed at 31–33°C with electrodes (3–5 MΩ) pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) filled with one of two intracellular solutions (in mM); (i) 135 CsMeSO4, 8 KCl, 4 MgATP, 0.3 NaGTP, 5 QX–314, 0.5 EGTA and 3 mg/ml biocytin. (ii) 150 K–gluconate, 3 $MgCl_2$, 0.5 EGTA, 2 MgATP, 0.3 Na2GTP, 10 HEPES and 3 mg/ml biocytin. pH was adjusted to 7.3 with KOH or CsOH and osmolarity adjusted to 285–295 mOsm. Whole–cell patch–clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Signals were filtered at 4 – 10 kHz and digitized at 10 – 20 kHz (Digidata 1322A and pClamp 10 Software; Molecular Devices, Sunnyvale, CA). For cell attached recordings pipettes were filled with our standard extracellular solution.

For optogenetic stimulation of ChR2 light stimuli at a wavelength of 470nm and duration of 1-5 ms were delivered through the 40X water immersion objective using a CoolLED pE–4000 Illumination system (Andover, UK). Arbitrary LED power typically ranged between 10 – 100% corresponding to ~ 0.4 – 6.9 mW/mm^2 as measured with a digital power meter (Thor Labs, NJ, USA). sEPSC analyses was performed on post hoc filtered episodic traces (Clampfit; Bessel 8-pole, -3db cutoff 1000Hz). Frequency of sEPSCs and average sEPSC amplitude were determined over 5 consecutive sweeps totalling a period of 4 seconds (i.e. 0.8s per sweep). sEPSC were detected via a thresholding procedure set at 4 times the RMS baseline noise. Note that neurons with a baseline sEPSC frequency of > 8Hz were selected for further interrogation. Analyses of electrophysiological data was performed in Clampfit (Molecular Devices, Sunnyvale, CA) and curated in Microsoft Excel spreadsheets.

Statistical Analyses

Tests for normality (Shapiro-Wilk test) were performed on all datasets to be compared prior to statistical analyses and appropriate parametric (unpaired or paired t-tests) or non-parametric (Mann-Whitney U or Wilcoxin signed rank tests) were accordingly performed. Exact p-values are stated within the Fig.s and values of $p<0.05$ or $p<0.01$ were deemed significant and designated with 1 or 2 asterisks, respectively. Error bars in the figures represent standard error of the means unless otherwise indicated. Box whisker plots were constructed as follows: symbol denotes mean value; line represents the median value; lower and upper box limits represent

25th and 75th percentiles, respectively. Lower and upper whiskers represent minimal and maximal data points, respectively.

AUTHOR CONTRIBUTONS

RC was responsible for project conceptualization, experimental design, electrophysiology experiments, image processing and manuscript writing and figure preparation. CMcB, RC, KAP supervised the project. XY, AV, EL and SH conducted IHC and imaging. DA and SH provided critical support for mouse breeding, genotyping and colony maintenance.

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COMPETING INTERESTS

The authors declare no competing interests.

This bioRxiv submission was coordinated with that of a study from the Hnasko laboratory (UCSD) entitled “Mu-opioid receptor activation potentiates excitatory transmission at the habenulo-peduncular synapse” by Singhal *et al.*

FIGURES

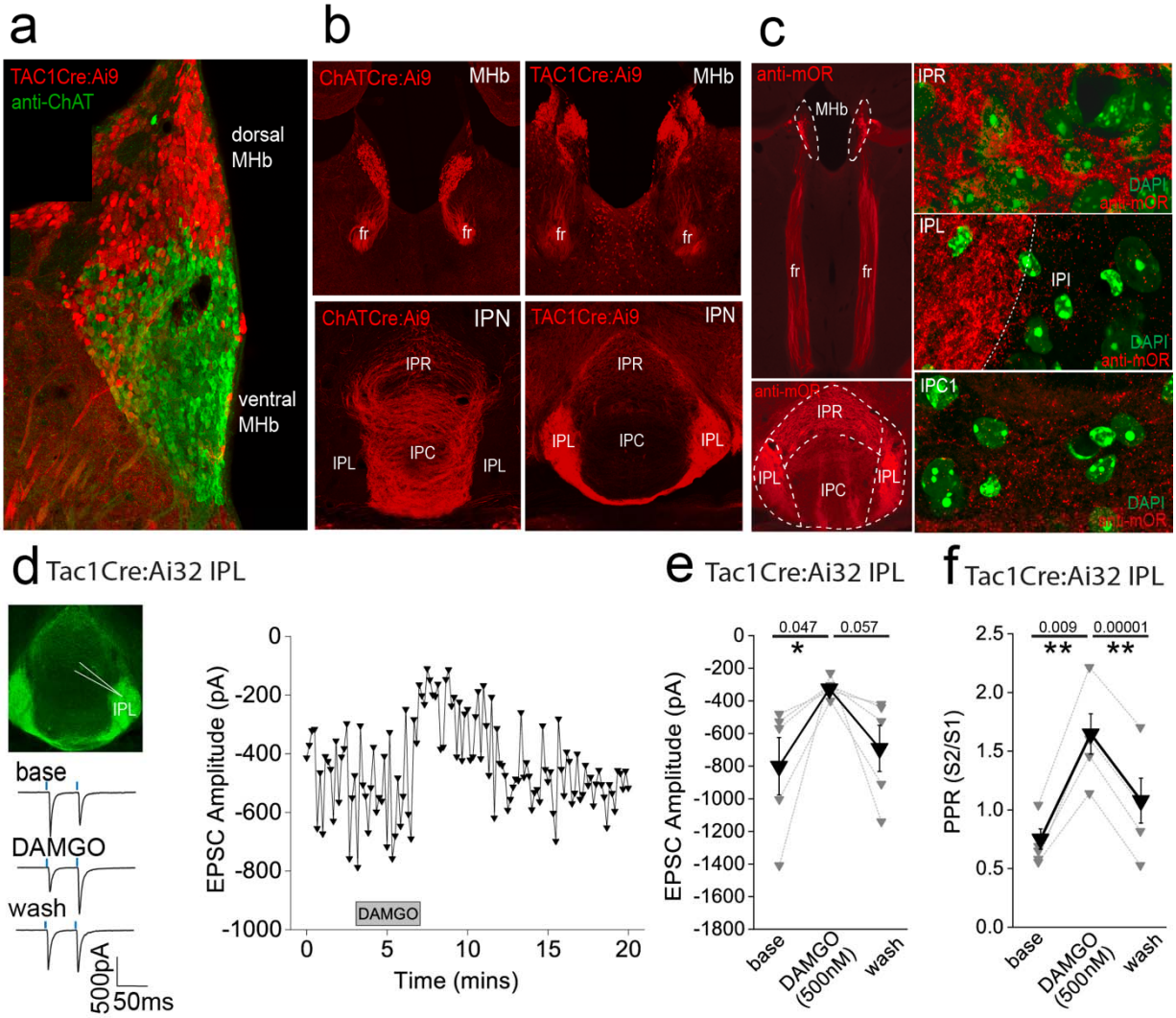


Fig. 1. AMPAR-mediated synaptic transmission in lateral IPN mediated by mHB substance P neurons is inhibited by mOR activation **(a)** Confocal image of a Tac1Cre:Ai9 mouse immunostained with anti-ChAT illustrating the distinct distribution of substance P (red) and cholinergic neurons (green) in dorsal and ventral mHb, respectively. **(b)** Conditional td-Tomato expression in cholinergic (left panels; ChATcre:Ai9) and SP neurons (right panels; Tac1Cre:Ai9) illustrating their axonal outputs via the fasciculus retroflexus (fr) and largely non-overlapping axonal arborization patterns in IPN. **(c)** Endogenous mOR expression throughout the mHb and IPN axis (left panels; red) assessed by immunocytochemistry. High resolution airy scan images of mOR distribution in subdivisions of the IPN; rostral IPN (IPR), lateral IPN (IPC) and central IPN (IPC). Green fluorescence are DAPI stained nuclei. **(d)** Whole-cell voltage-clamp in adult (>p40) Tac1Cre:Ai32 mice (top left panel illustrating the axonal arborization of ChR2 expressing SP neuronal axons in IPN and the position of neuronal recording in IPL). Single voltage-clamp traces of light evoked AMPAR EPSCs (bottom left panel; 470nM light pulse; 2 stimulations at 20Hz, blue dashes)) and time course of peak amplitudes (right panel) under baseline, during application of 500 nM DAMGO and washout conditions. **(e,f)** Individual (grey filled symbols) and mean (black filled symbols) data of AMPAR EPSC amplitude and corresponding paired pulse ratios (PPR; n=5 recorded IPL neurons from 5 mice).

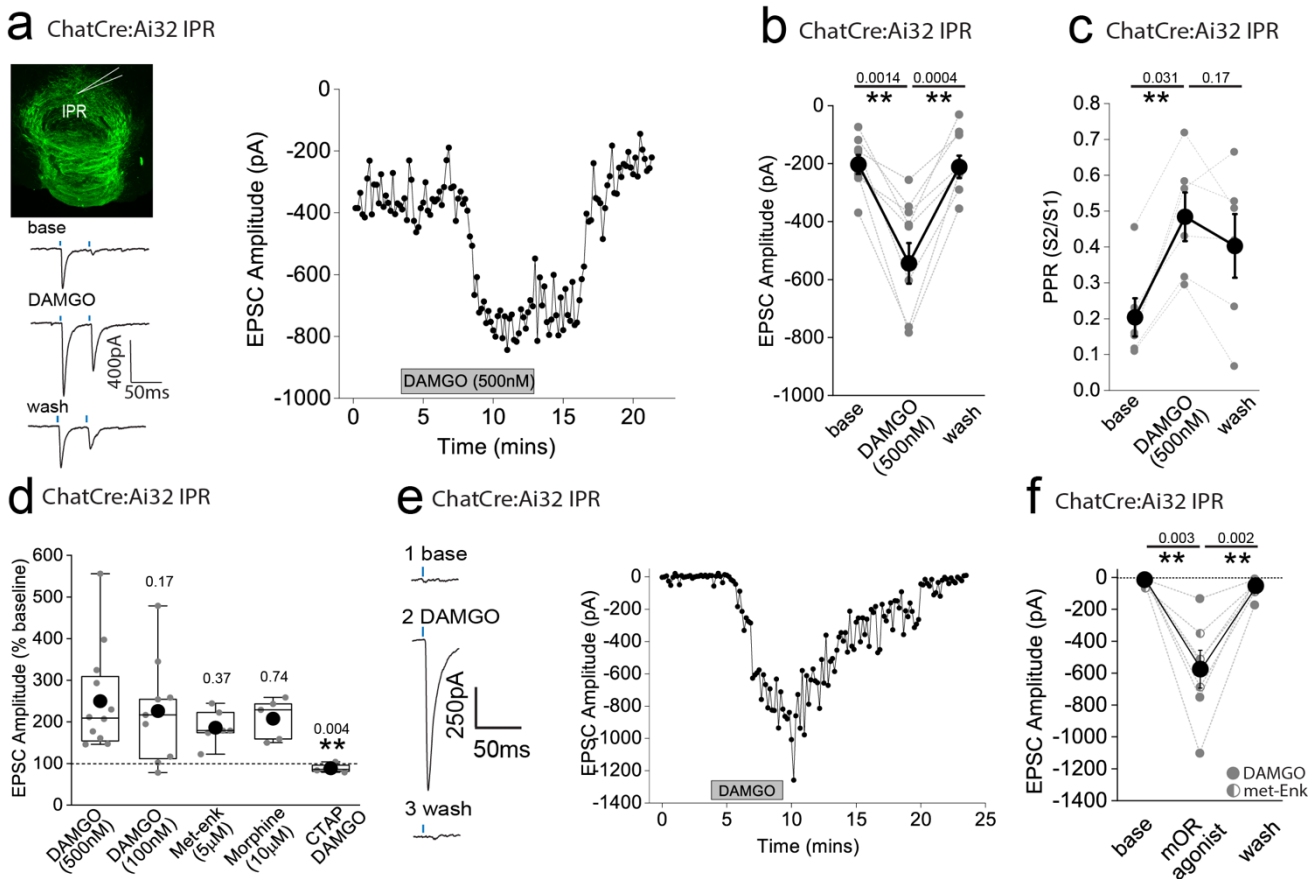


Fig. 2. AMPAR-mediated synaptic transmission in rostral IPN mediated by mHB cholinergic neurons is potentiated by mOR activation **(a)** Whole-cell voltage-clamp in adult (>p40) ChATCre:Ai32 mice (top left panel illustrating the axonal arborization of ChR2 expressing SP neuronal axons in IPN and the position of neuronal recording in IPR). Single voltage-clamp traces of light evoked AMPAR EPSCs (bottom left panel; 470nM light pulse; 2 stimulations at 20Hz, blue dashes) and time course of peak amplitudes (right panel) under baseline, during application of 500 nM DAMGO and washout conditions. **(b,c)** Individual (grey filled symbols) and mean (black filled symbols) data of AMPAR EPSC amplitude and corresponding paired pulse ratios (PPR; n=8 recorded IPR neurons from 7 mice). **(d)** Summary bar graph illustrating the effect of DAMGO (500nM and 100nM; n=8 recorded IPR neurons from 7 mice and n=9 recorded IPR neurons from 4 mice, respectively), met-enkephalin (5μm; n=7 recorded IPR neurons from 5 mice), morphine (10μM; n=5 recorded IPR neurons from 5 mice) and 500nM DAMGO in presence of mOR antagonist 1mM CTAP (n=4 recorded IPR neurons from 2 mice). **(e)** Voltage-clamp trace examples (single light stimulus; blue bar; left panel) and time course (right panel) under baseline, DAMGO and washout conditions in a recorded IPR neuron displaying no measurable baseline AMPAR EPSC in response to maximal light evoked stimulation (470nm; 6.9mW/mm^2). **(f)** Individual (gray filled and half-filled symbols for 500nM DAMGO or 5μM Met-enkephalin respectively) and pooled (black filled symbols) data of light-evoked AMPAR EPSC amplitude during DAMGO application and washout (n = 4 and 3 recorded IPR cells from 4 and 3 mice for DAMGO and met-enkephalin application, respectively).

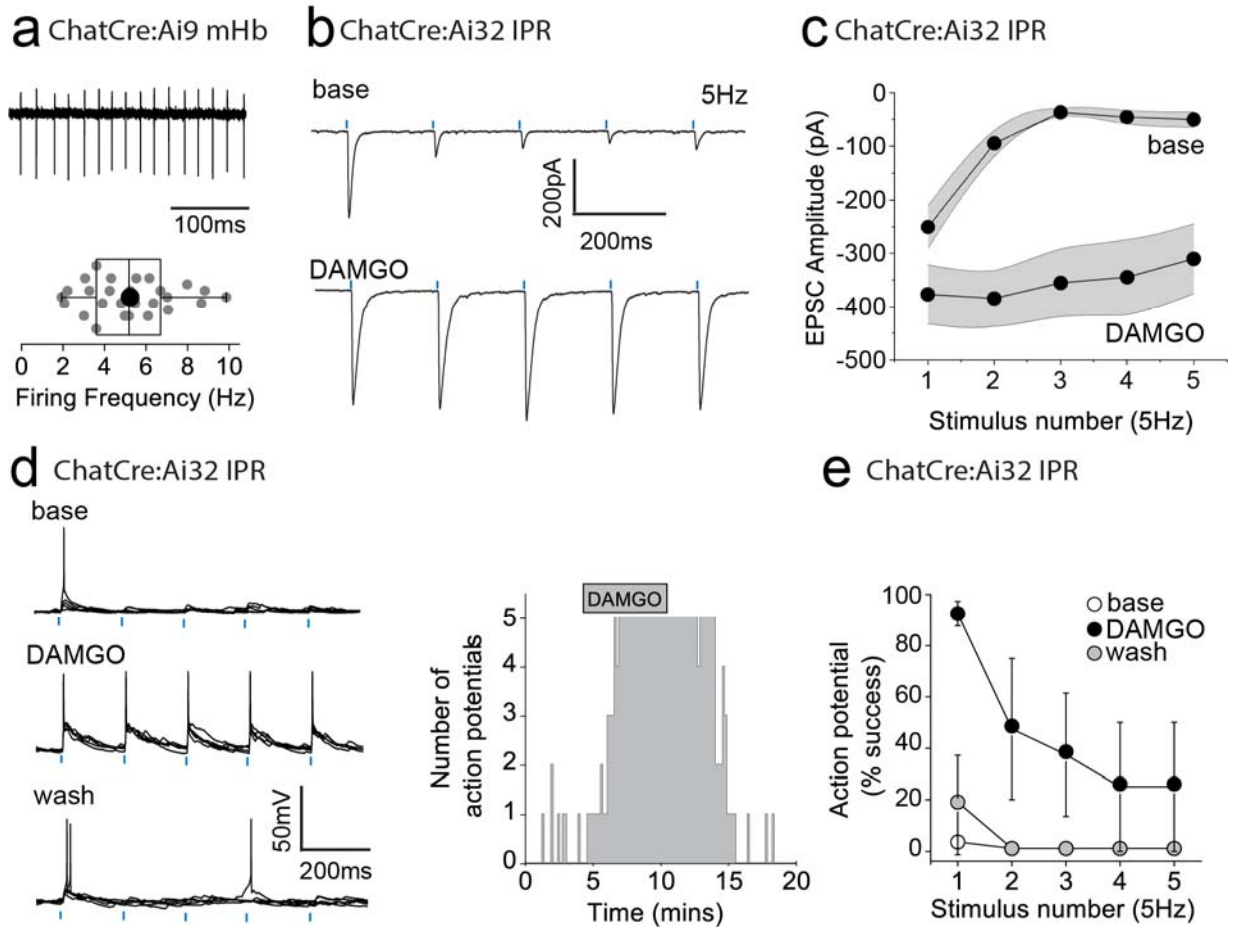


Fig. 3. mOR activation increases fidelity of glutamatergic transmission mediated by mHb cholinergic neurons to augment excitation:spike coupling in postsynaptic IPR neurons. **(a)** Single example trace showing spontaneous action potential firing in cell attached mode from a td-Tomato-positive ventral mHb neuron in the ChatCre:Ai9 mouse (top panel). Box plot and corresponding individual data of the spontaneous firing frequency of mHB cholinergic neurons (bottom panel; n = 27 recorded cells). **(b)** Single voltage-clamp traces of light-evoked (470nm, 5 pulses delivered at 5Hz; blue dashes) AMPAR EPSCs recorded from postsynaptic IPR neurons in adult ChATCre:Ai32 mice (p>40) under baseline and following 500nM DAMGO application (left panel). **(c)** Pooled data (shaded area denotes SEM) of peak amplitude for each given stimulus in the 5Hz train during baseline and following 500nM DAMGO application (n = 5 recorded IPR neurons from 5 mice). **(d)** Single current-clamp traces example (5 consecutive overlaid sweeps) of light-evoked EPSP:action potential coupling (5 stimuli at 5 Hz; blue dashes) under baseline, 500nM DAMGO and washout conditions (left panel). Corresponding single example time course plot depicting number of light-driven EPSP-evoked action potential (right panel). **(e)** Mean data showing percentage success over 5 consecutive traces of EPSP:action potential coupling for each given stimulus in the 5Hz train under baseline (open symbols), 500nM DAMGO (black symbols) and washout (grey symbols) conditions (n = 7 recorded IPR neurons from 6 mice).

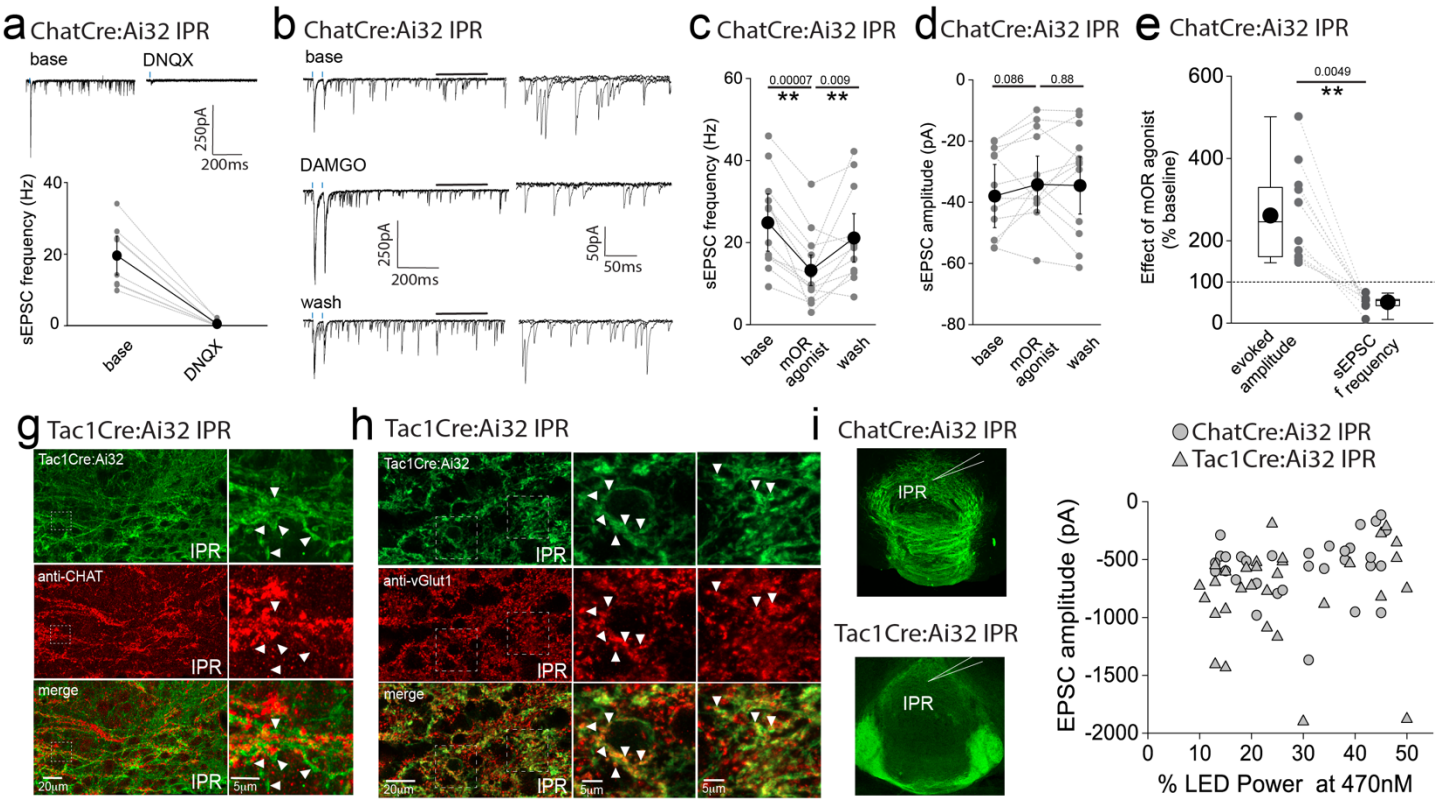


Fig. 4. mOR activation imparts opposing effects on evoked versus spontaneous AMPAR EPSCs mediated by mHB cholinergic neurons and demonstration of functional SP neuronal mediated evoked AMPAR EPSCs in IPR. (a) Single voltage-clamp traces (top panels) illustrating light-evoked AMPAR EPSCs mediate by cholinergic neurons and spontaneous EPSCs (sEPSCs) under baseline and following 10µM DNQX application in an IPR neuron. Individual (grey symbols) and pooled data (black symbols) illustrating complete cessation of sEPSCs as assessed by frequency following 10µM DNQX application (n = 8 recorded IPR neurons from 8 mice; bottom panel). **(b)** Single voltage-clamp traces under baseline, 500nM DAMGO and washout conditions of light-evoked AMPAR EPSCs mediated by mHb cholinergic neurons (2 stimuli at 20Hz; blue dashes) and sEPSCs (left panels). Magnification of sEPSCs events (right panels) corresponding to the region of the traces in the left panels delineated by the black bars. **(c,d)** Individual (grey symbols) and mean (black symbols) data of sEPSC frequency and amplitude (n = 12 recorded IPR cells from 11 mice). **(e)** Box plot and individual data depicting the relative DAMGO mediated percentage change from baseline of the light-evoked AMPAR EPSC peak amplitude versus AMPAR sEPSC frequency in each individual IPR neuron recorded (n = 12 recorded IPR cells from 11 mice). **(g)** High resolution airy scan of the IPR in TAC1Cre:Ai32 mouse showing ChR2-expressing synaptic bouton like-structures (green) and endogenous ChAT expression (red). Right panels are magnified regions of the boxed area. Arrows indicate examples of non-overlap of TacCre:Ai32 boutons with ChAT. **(h)** High resolution airy scan of the IPR in TAC1Cre:Ai32 mouse showing ChR2-expressing synaptic bouton like-structures (green) and endogenous VGlut1 expression via immunostaining (red). Right panels are magnified regions of the boxed area. Arrows indicate examples of expression of VGluT1 within TacCre:Ai32 bouton structures. **(i)** Comparison of light-evoked AMPAR EPSC peak amplitude in postsynaptic IPR neurons mediated by either cholinergic (ChATCre:Ai32 mice; n = 32 recorded IPR neurons) and substance P (Tac1Cre:Ai32 mice; n = 31 recorded IPR neurons) mHB neurons at various arbitrary % LED (10-50% corresponding to an approximate power of 0.4 – 3.4 mW/mm^2; right panel).

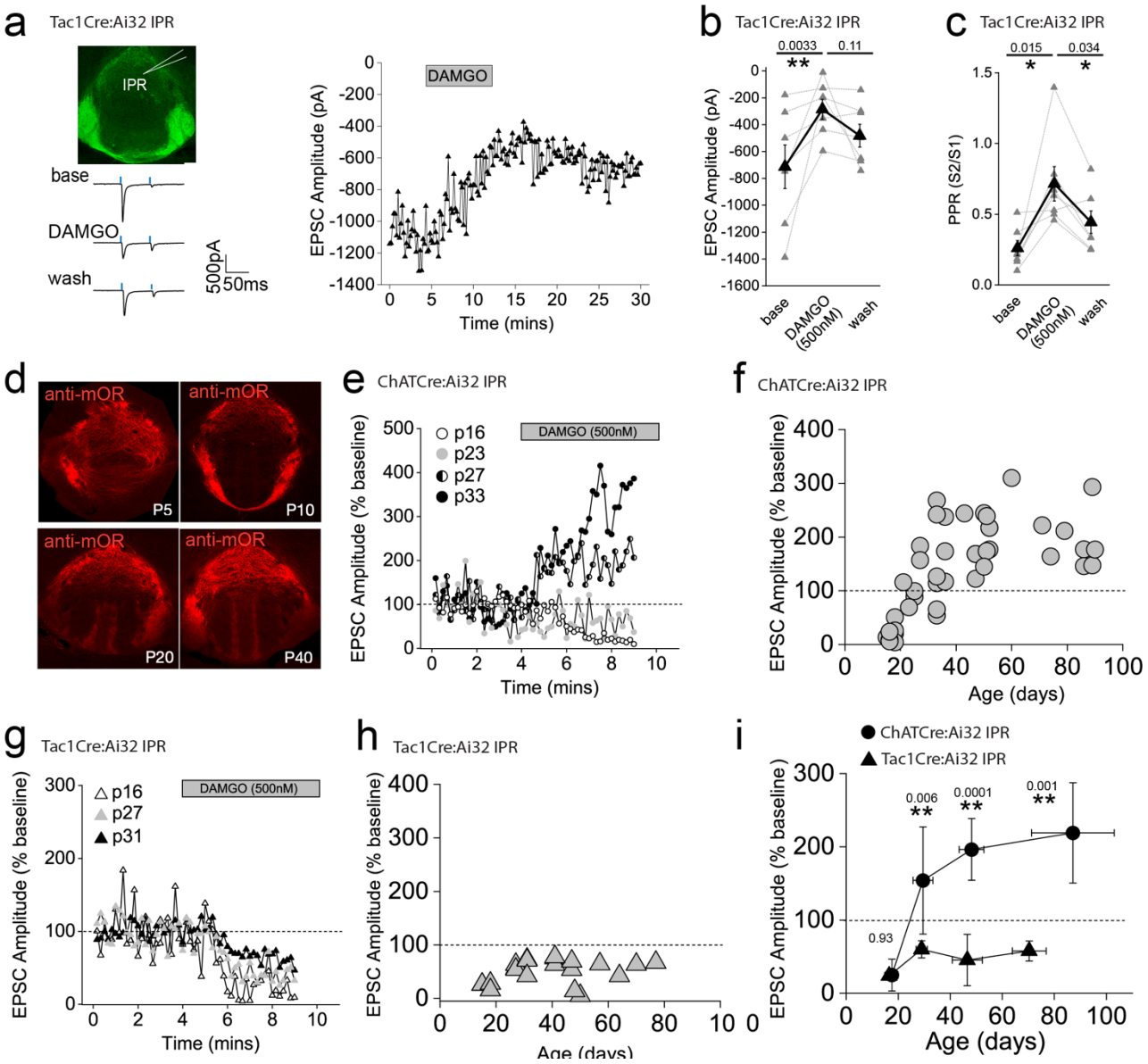


Fig. 5. mORs constitute a developmentally regulated molecular switch altering the salience of neurotransmission in IPR mediated by substance P (SP) versus cholinergic neurons (a) Whole-cell voltage-clamp in adult (>p40) TAC1Cre:Ai32 mice (top left panel illustrating the axonal arborization of ChR2 expressing SP neuronal axons in IPN and the position of neuronal recording in IPR). Single voltage-clamp traces of light evoked AMPAR EPSCs (bottom left panel; 470nM light pulse; 2 stimulations at 20Hz, blue dashes) and time course of peak amplitudes (right panel) under baseline, during application of 500 nM DAMGO and washout conditions (right panel). **(b,c)** Individual (grey filled symbols) and mean (black filled symbols) data of AMPAR EPSC amplitude and corresponding paired pulse ratios (PPR; n=7 recorded IPR neurons from 7 mice). **(d)** Confocal images of mOR protein expression in IPN during development (p5-p40). **(e)** Single examples of the time course of light-evoked AMPAR EPSC peak amplitudes mediated by mHB cholinergic neurons following DAMGO application in postsynaptic IPR neurons at varying ages as indicated. **(f)** Pooled data of the percent change of light-evoked cholinergic neuronal mediated EPSC peak amplitude elicited following DAMGO application across all ages tested (n = 44 recorded IPR neurons). **(g)** Single examples of the time course of light-evoked AMPAR EPSC peak amplitudes mediated by mHB substance P neurons following DAMGO application in postsynaptic IPR neurons at varying ages as

indicated. **(h)** Pooled data of the percent change of light-evoked SP neuronal mediated EPSC peak amplitude elicited following DAMGO application across all ages tested. **(i)** Summary plot of the mean changes in the normalized AMPAR EPSC peak amplitude (% baseline) by DAMGO mediated by cholinergic and SP neurons binned at the following developmental epochs; p15-23 (postnatal), p24-34 (adolescent/pre-pubescent), p35-60 (adolescent/pubescent, sexual maturation) and >p60 (adult)[91]. Error bars denote standard deviation of the mean. Note panels f,g and h,i contain normalized data from the same recorded cells depicted in Figs. 2b and 5b as absolute peak amplitude changes, respectively.

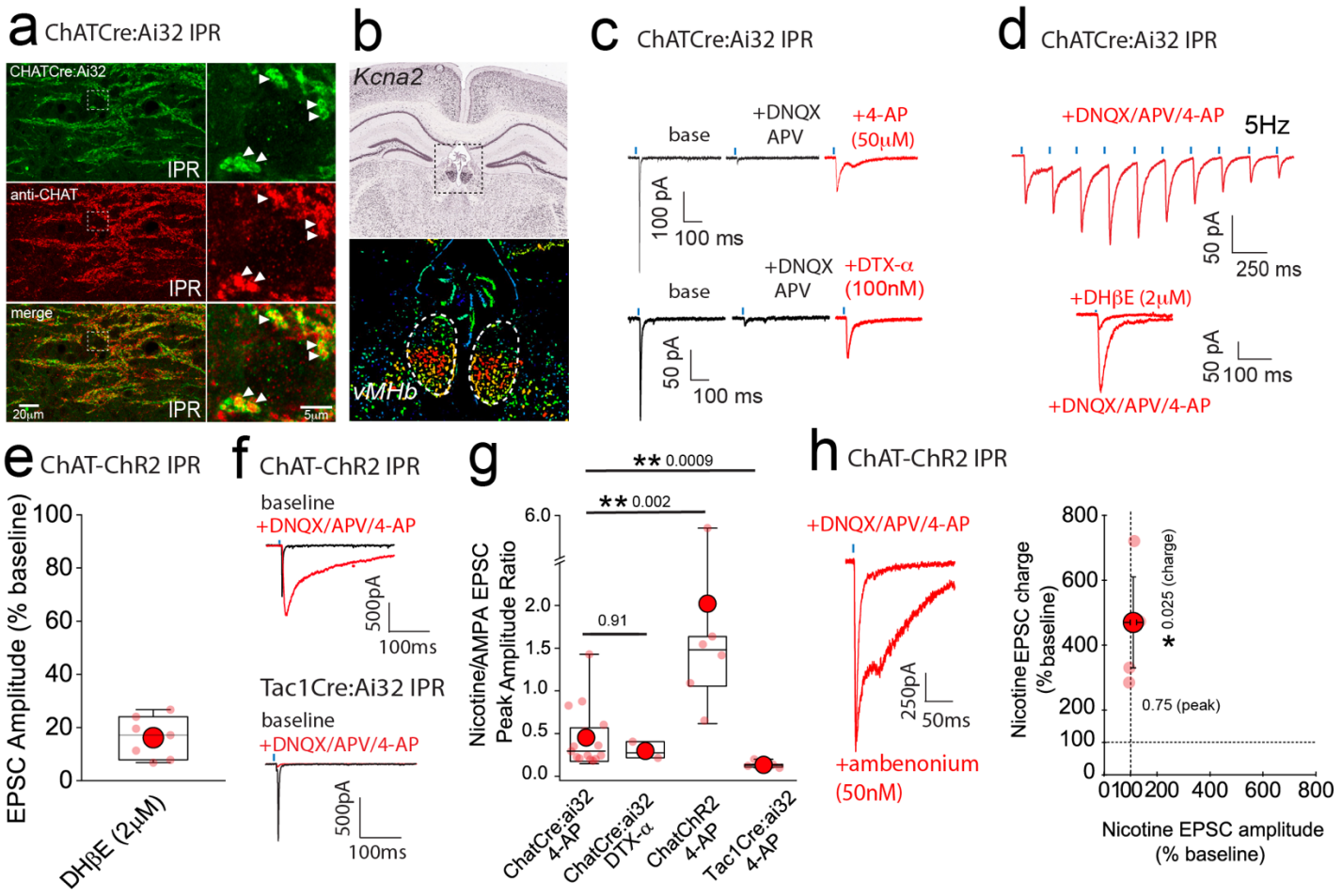


Fig. 6. Kv1 channels constitute a molecular brake of nicotinic receptor mediated signaling in the IPN. **(a)** High resolution airy scan images of IPR in ChATCre:ai32 mice showing ChR2-expressing cholinergic boutons (green) and endogenous ChAT (red). Right panels are magnified regions of the boxed area in left panels. Arrows indicate faithful expression of ChAT within ChatCre:Ai32 boutons (cf. Figure 4g). **(b)** *In situ* hybridization for *KCNA2* mRNA (top panel) and corresponding pseudo-colored expression level (bottom panel) illustrating bias towards ventral mHb. Data are from the Allen Brain Institute (https://mouse.brain-map.org/gene/show/16263). **(c)** Voltage-clamp example traces of light-evoked EPSCs mediated by cholinergic mHb neurons (left panel) under baseline, following 10µM DNQX/100µM DL-APV plus 50µM 4-AP (top panel) or plus 100nM dendrotoxin-α (bottom panel). **(d)** Voltage-clamp example trace of light-evoked EPSCs mediated by cholinergic mHb neurons in the presence of 10µM DNQX/100µM DL-APV/100µM 4-AP. Light delivered at 5Hz for 10 stimuli under baseline conditions (top panel) or 1 stimulus in the absence or presence of 2µM DHβE (bottom panel). **(e)** Box plot with individual data of the percentage inhibition of the light-driven EPSC peak amplitude mediated by cholinergic neurons in the presence of 10µM DNQX/100µM DL-APV/50µM 4-AP (n = 7 recorded IPR neurons from 7 mice). **(f)** Voltage-clamp example trace of light-evoked EPSCs under baseline and after addition of 10µM DNQX/50µM DL-APV/100µM 4-AP in ChAT-ChR2 (top panel) and Tac1Cre:ai32 (bottom panel). **(g)** Box plot with individual data of the nicotine/AMPA peak amplitude ratio within individual recorded IPR neurons percentage as measured under baseline (AMPA EPSC) and in the presence of 10µM DNQX/50µM DL-APV/100µM 4-AP (nAChR EPSC; n = 15 recorded IPR neurons from 15 ChATCre:Ai32 mice) or 100nM dendrotoxin-α (nAChR EPSC; n = 3 recorded IPR neurons from 3 ChATCre:Ai32 mice). nAChR/AMPA peak EPSC amplitude ratios were also performed in ChAT-ChR2 (n = 6

recorded IPR neurons from 6 mice) and Tac1Cre:ai32 mice (n = 6 recorded IPR neurons from 6 mice). **(h)** Voltage-clamp example trace of light-evoked nAChR-mediated EPSCs mediated by cholinergic mHb neurons under baseline and following application of 50nM ambenonium (left panel). Scatter plot of the individual (grey symbols) and pooled (red symbol) percentage change in nAChR EPSC peak amplitude versus EPSC charge (measured over the first 500ms duration of the EPSC) in each individual recording (n = 4 recorded IPR neurons in 4 mice; right panel).

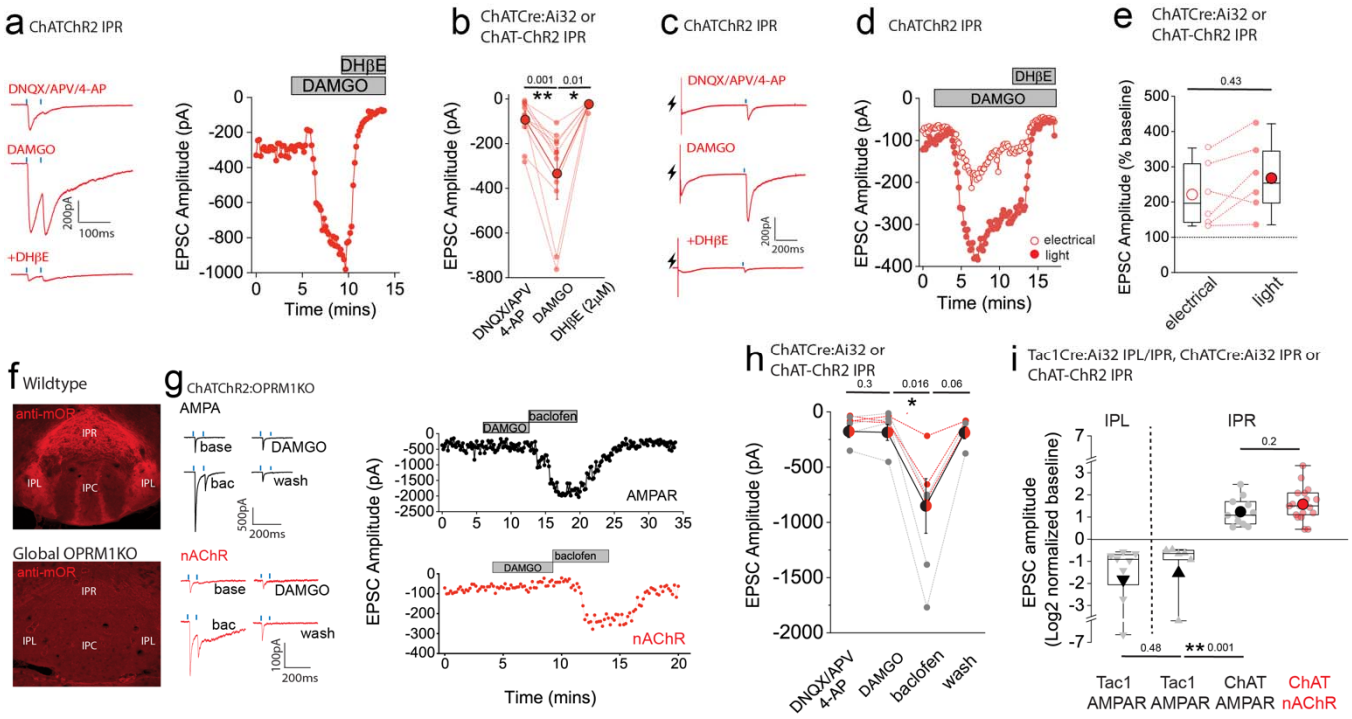
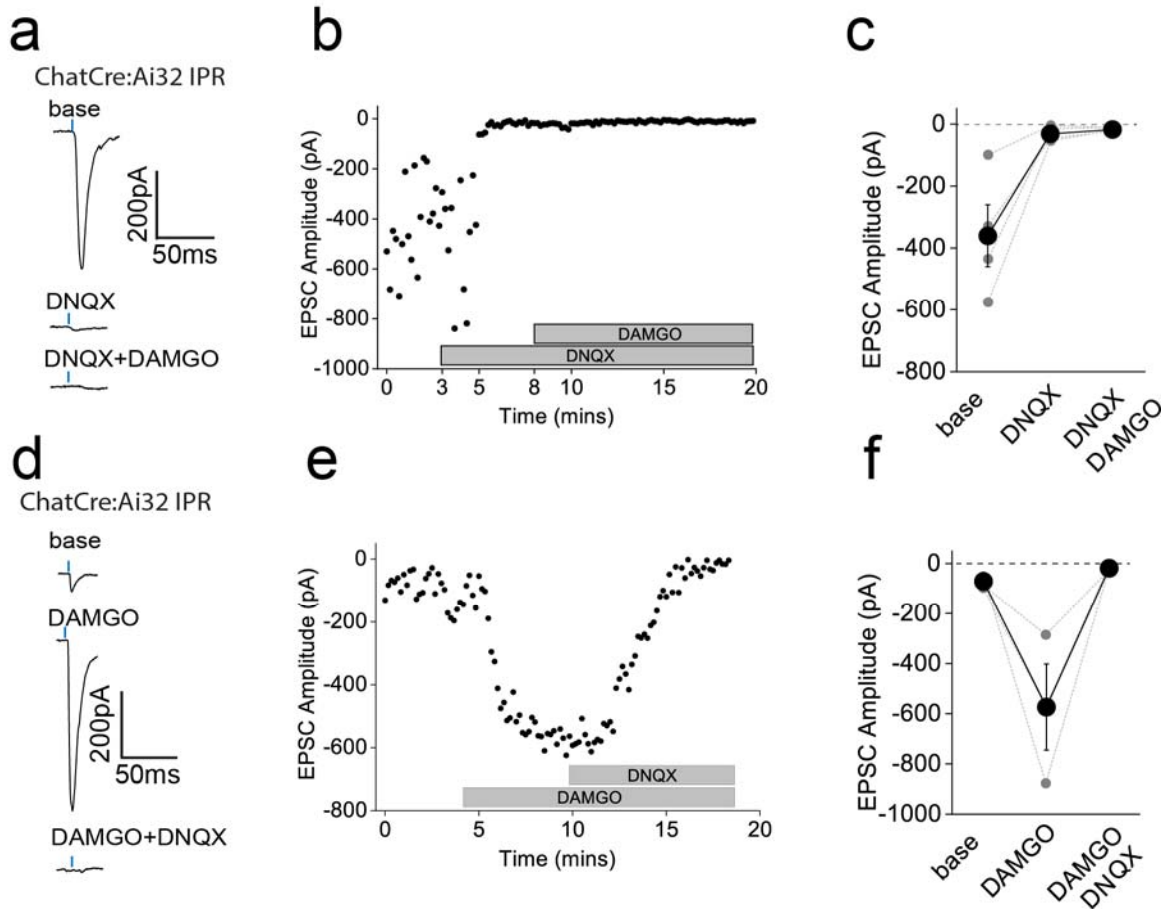


Fig. 7. mOR potentiates nAChR EPSC amplitude revealing an interplay between opioid and cholinergic systems in the habenulo-interpeduncular axis. (a) Voltage-clamp example traces of light-evoked nAChR EPSCs (2 stimuli at 20Hz; blue dashes) mediated by cholinergic mHb neurons (left panel) and time course of peak amplitude (right panel) under baseline, 500nM DAMGO and 500nM DAMGO plus 2μm DHβE conditions. **(b)** Individual and mean (red filled symbols) data of nAChR EPSC peak amplitude. **(c,d)** Voltage-clamp example traces of simultaneous electrical (lightning symbol) and light-evoked (blue dash) nAChR EPSCs and time course of peak amplitude (open and filled symbols representing electrical and light-evoked peak amplitude of nAChR EPSCs, respectively) under baseline, 500nM DAMGO and 500nM DAMGO plus 2μm DHβE conditions. **(e)** Percentage change in electrical and light-evoked nAChR EPSC peak amplitude elicited by 500nM DAMGO in each individual recorded IPR neuron (n = 6 recorded IPR neurons from 6 mice). **(f)** Confocal images of endogenous mOR protein expression in IPN of WT and homozygote OPRM1 KO mice. **(g)** Voltage-clamp example traces of light-evoked AMPA and nAChR EPSCs (2 stimuli at 20Hz; blue dashes) mediated by cholinergic mHb neurons (top and bottom left panels, respectively) and time course of peak amplitude (right panels) under baseline, 500nM DAMGO, 1μM baclofen and washout conditions in ChATCHR2:OPRM1KO mice. **(h)** Individual and mean data of light-evoked AMPAR (black symbols; n=4 from 4 mice) and nAChR (red symbols; n=3 from 3 mice) peak amplitude in response to DAMGO and baclofen application in ChATCHR2:OPRM1KO mice. Note in 2 of the 4 AMPAR EPSC recordings washout of the baclofen effect was not performed). **(i)** Summary box plot with individual data of the percentage normalized changes (log2) of AMPAR and nAChR peak amplitudes in response to mOR activation mediated by SP and cholinergic neurons in various subdivisions of IPN tested. Note that the data in this panel are replotted from the absolute peak EPSC amplitude changes mediated by mOR agonists in Figs. 1e, 2b, 5a and 7b.



Supplementary Figure 1. mOR induced potentiation of light-driven EPSCs by mHb cholinergic neurons are mediated exclusively by AMPARs.
(a) Voltage-clamp example traces of light-evoked AMPAR EPSCs mediated by cholinergic mHb neurons (left panel) and time course of peak amplitude (right panel) under baseline, 10µM DNQX and 10µm DNQX plus 500nM DAMGO conditions. **(c)** Individual (grey filled symbols) and mean (black filled symbols) data of AMPAR EPSC amplitude under baseline, 10µM DNQX and 10µm DNQX plus 500nM DAMGO conditions (n = 4 recorded IPR neurons from 4 mice). **(c)** Voltage-clamp example trace of single light-evoked AMPAR mediated EPSCs mediated by cholinergic mHb neurons (left panel) and time course of peak amplitude (right panel) under baseline, 500nM DAMGO and 500nM DAMGO plus 10µm DNQX conditions. **(d)** Individual (grey filled symbols) and mean (black filled symbols) data of AMPAR EPSC amplitude under, baseline, 500nM DAMGO and 500nM DAMGO plus 10µm DNQX conditions. (n = 3 recorded IPR neurons from 3 mice).