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Two delta opioid receptor subtypes are functional in single ventral tegmental area neurons, and can interact with the mu opioid receptor

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[D-Ala^2, N-Me-Phe^4, Gly-ol^5]-Enkephalin acetate salt (DAMGO) (PubChem CID: 5462471)
D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) (PubChem CID: 10418702)
H-Tyr-Tic-psi(CH_2NH)Phe-Phe-OH (TIPP-psi) (PubChem CID: 5311481)

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ABSTRACT

The mu and delta opioid receptors (MOR and DOR) are highly homologous members of the opioid family of GPCRs. There is evidence that MOR and DOR interact, however the extent to which these interactions occur *in vivo* and affect synaptic function is unknown. There are two stable DOR subtypes: DPDPE sensitive (DOR1) and deltorphin II sensitive (DOR2); both agonists are blocked by DOR selective antagonists. Robust motivational effects are produced by local actions of both MOR and DOR ligands in the ventral tegmental area (VTA). Here we demonstrate that a majority of both dopaminergic and non-dopaminergic VTA neurons express combinations of functional DOR1, DOR2, and/or MOR, and that within a single VTA neuron, DOR1, DOR2, and MOR agonists can differentially couple to downstream signaling pathways. As reported for the MOR agonist DAMGO, DPDPE and deltorphin II produced either a predominant K^+ dependent hyperpolarization or a $Ca_v2.1$ mediated depolarization in different neurons. In some neurons DPDPE and deltorphin II produced opposite responses. Excitation, inhibition, or no effect by DAMGO did not predict the response to DPDPE or deltorphin II, arguing against a MOR-DOR interaction generating DOR subtypes. However, in a subset of VTA neurons the DOR antagonist TIPP-Ψ augmented DAMGO responses; we also observed DPDPE or deltorphin II responses augmented by the MOR selective antagonist CTAP. These findings directly support the existence of two independent, stable forms of the DOR, and show that MOR and DOR can interact in some neurons to alter downstream signaling.

1. Introduction

Opiate drugs and endogenous opioid peptides exert powerful behavioral actions through binding to receptors expressed on neurons in the central nervous system. These opioid receptors belong to family A of G protein-coupled receptors (GPCRs), exhibit high amino acid sequence homology in their transmembrane domains, and control neuronal activity through similar intracellular signaling pathways and ionic conductances. Of particular interest in this regard are the mu opioid receptor (MOR) and the delta opioid receptor (DOR) as they have the highest amino acid sequence homology (Chang et al., 2004), are often expressed in high density in the same brain regions (Erbs et al., 2015), and respond to similar concentrations of the endogenous opioid peptides leucine enkephalin (l-enk), methionine enkephalin (m-enk) and β-endorphin (Chang et al., 2004). Despite these molecular and cellular similarities, MOR and DOR agonists can generate different and often opposing effects on motivated behaviors (e.g. analgesia, reward, motivation) (Bals-Kubik et al., 1993; Farias et al., 2003; Hammond et al., 1998; Margolis et al., 2008a; Mitchell et al., 2014).

Complicating our understanding of the interaction between MOR and DOR is the evidence that there are two consistent functional forms of DOR: DOR1, selectively activated by the synthetic cyclic peptide [D-Pen^2, D-Pen^5]enkephalin (DPDPE) (Mosberg et al.,

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1983) and DOR2, selectively activated by the amphibian skin derived peptide [D-Ala^2, Glu^4]deltorphin (deltorphin II) (Erspamer et al., 1989; Kreil et al., 1989). There is also evidence for partial selectivity of antagonists: 7-benzylidenenaltrexone (BNTX) preferentially blocks DOR1 activity (Sofuoglu et al., 1993), and naltriben and 5′NTII preferentially block DOR2 actions (Jiang et al., 1991; Portoghese et al., 1991; Sofuoglu et al., 1991). However, the pharmacologic properties of these antagonists seem to be tissue dependent (Zaki et al., 1996). Further evidence for distinct actions of the two DOR forms is that in rodents both DPDPE and deltorphin II produce analgesia, however repeated exposure to either ligand does not produce cross-tolerance to the other DOR agonist (Mattia et al., 1991). Clearly, unraveling the neurobiological underpinnings of these distinct responses has the potential to improve the clinical success of DOR based therapeutics.

One proposed explanation for these DOR subtypes is receptor heterodimerization (van Rijn and Whistler, 2009). Opioid receptors typically signal through $G_{i/o}$ proteins, inhibiting adenylyl cyclase, opening K^+ channels to inhibit firing or closing Ca^{2+} channels to decrease neurotransmitter release (Williams et al., 2001). The resolution of the crystal structures of MOR and DOR supports the possibility of direct interaction: the receptors crystallized as dimers, interfacing at molecular domains that are virtually identical between MORs and DORs (Manglik et al., 2012; Provasi et al., 2015). In cultured cells MOR-DOR heterodimerization can change the intracellular signaling properties of MOR or DOR ligands, conferring a preferential coupling to non-G protein mediated signaling pathways (Rozenfeld and Devi, 2007). Heterodimerization of MORs and DORs can also generate an unexpected agonist-antagonist interaction observed in heterologous expression systems, such that a DOR antagonist *increases* the potency and efficacy of a MOR agonist and vice-versa at G protein dependent pathways (Gomes et al., 2000, 2011), presumably by enabling the heterodimers to switch from G protein independent to G protein dependent signaling. This type of interaction at the neuronal level *in vivo* would complicate the interpretation of data from behavioral pharmacology experiments that use receptor selective antagonists, since it raises the possibility that an antagonist will not just block activation of the intended receptor, but may also increase the efficacy or potency of an endogenous peptide acting at a heterodimer receptor partner. Further evidence for functional MOR-DOR heterodimers is that synthetic bivalent compounds that combine MOR agonist and DOR antagonist actions show enhanced MOR analgesia and reduced MOR tolerance, dependence, and reward (Daniels et al., 2005). The atomic spacing between the MOR agonist and DOR antagonist components of the bivalent molecule is critical (must be greater than 22 Å), suggesting that the ligand's action depends on MOR and DOR binding sites being a specific and relatively short distance from each other.

Another possibility is that the behavioral differences observed in response to DOR subtype pharmacologies is generated by functional selectivity or biased agonism. That is, structurally distinct DOR selective ligands induce different conformational changes in the same receptor that favor activation of one or another intracellular signaling pathway, thereby imposing different effects on the circuit. The first evidence that such ligand-directed alternative signaling is possible was demonstrated in studies of the β2-adrenergic receptor (Drake et al., 2008). Also, as heterodimerized receptors signal through alternative mechanisms, a heterodimer-selective ligand (Fujita et al., 2014; Gomes et al., 2013) would appear to be a biased agonist. Splice variants may also lead to different pharmacologies (Pasternak, 2001), however it is unknown if DOR splice variants are expressed in neurons and have functional consequences. While each of these possibilities for functional diversity depends upon ligand-receptor and receptor-receptor interaction within a single cell, to date, there has been no direct demonstration that the pharmacological differences between DOR1 and DOR2 ligands at the behavioral level can be explained by different molecular interactions at the single neuron level.

DOR1 activation in the ventral tegmental area (VTA) increases dopamine release in the nucleus accumbens (NAc) (Devine et al., 1993a, 1993b). Although DPDPE does not induce a conditioned place preference when infused into the VTA (Mitchell et al., 2014), animals will self-administer DPDPE directly into the VTA, suggesting that, like MOR activation, DOR1 activation in the VTA has a positive motivational effect (Devine and Wise, 1994). However, in long term alcohol drinking rats, while the MOR selective antagonist CTAP reduces alcohol consumption, the DOR selective antagonist TIPP-Ψ increases it (Margolis et al., 2008a). These complex behavioral effects of selective DOR ligands in the VTA and the evidence of MOR-DOR competitive interaction contrast with the limited number of *ex vivo* electrophysiologic studies investigating actions of selective DOR agonists. For instance, an early study with a small sample size (3 neurons) found that DPDPE did not elicit a postsynaptic GIRK response (Johnson and North, 1992a). While a number of studies have used the endogenous opioid peptide m-enk to characterize MOR actions in the VTA (e.g. (Ford et al., 2006; Johnson and North, 1992b)), m-enk also acts at DOR. MOR activation by the selective agonist DAMGO induces robust presynaptic inhibition of GABA release in VTA but we have detected only small DOR effects on GABA release in EtOH naïve animals (Margolis et al., 2008a; Mitchell et al., 2014). Because MOR and DOR in the VTA elicit robust motivational and rewarding actions and because DOR1 and DOR2 agonists in the VTA can differ in their synaptic and behavioral actions (Margolis et al., 2008a; Mitchell et al., 2014), we investigated DOR subtype function and interactions of DOR with MORs in single neurons from throughout the VTA; we characterized the postsynaptic responses to DPDPE and deltorphin II, compared these to responses to the MOR agonist DAMGO, and probed for MOR-DOR interactions.

2. Methods

2.1. Animals

Animal care and all experimental procedures were in accordance with guidelines from the National Institutes of Health and approved in advance by the Ernest Gallo Clinic (through June 2013) and Research Center and the University of California, San Francisco Institutional Animal Care and Use Committees (after July 2013).

2.2. Slice preparation and electrophysiology

Recordings were made in control male Sprague-Dawley rats (p22 to adult). 11% of recordings were completed in rats greater than 60 days old, including experiments of all types. No differences were observed between younger and adult animals, so the data are presented together. Some data were obtained in neurons also used for previously reported experiments (Margolis et al., 2014, 2006b, 2012). Rats were deeply anesthetized with isoflurane and then decapitated. Horizontal brain slices (150 μm thick) were prepared using a vibratome (Leica Instruments). Slices were prepared in ice cold Ringer solution (in mM: 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, and 11 glucose saturated with 95% O_2-5% CO_2) and allowed to recover at 33–35 °C for at least 1 h. Slices were visualized under a Zeiss Axioskop or Axioskop FS 2 plus with differential interference contrast optics and infrared illumination or an Axio Examiner A1 also equipped with Dodt optics, using a Zeiss Axiocam MRm and Axiovision 4 (Zeiss) or Microlucida (MBF Biosciences, Williston, VT, USA) software. Whole cell