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Author
Margolis, Elyssa B.

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Elyssa B. Margolis

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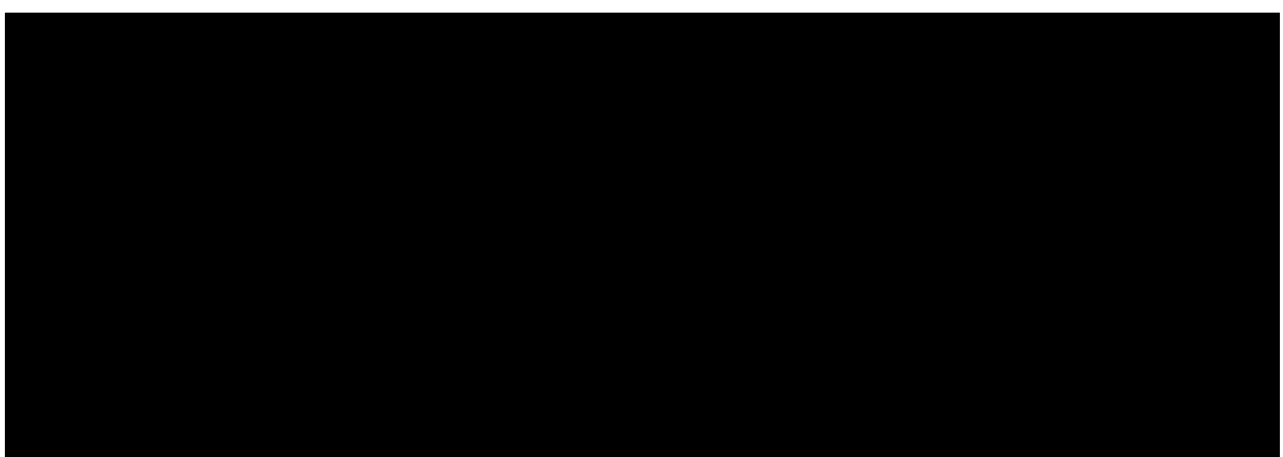

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Howard: Thank you for your guidance, generosity, friendship, and respect.

The text of Chapter 2 of this thesis is a reprint of the material as it appears in the Journal of Neuroscience November 5, 2003, 23(31):9981-9986.

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This work was carried out by Ms. Margolis. It represents a great deal of work. The histological methods were worked out by Ms. Margolis and some of the processing was carried out by Kelly Pollak, a technician working under the supervision of Ms. Margolis. Drs. Bonci, Hjelmstad and I served as technical advisors on the project, with Dr. Bonci providing expertise in the preparation and literature on the ventral tegmental area and Dr. Hjelmstad helping with preparation of slices, setting up equipment and writing and using data analytic software. My contribution was in the selection of the project, research design and strategy for combining the different techniques that were used. The combination of whole cell recording, labeling recorded cells and immunohistochemistry represents a remarkable technical achievement and in scope and data is more than comparable to the portion of a standard thesis that it replaces.

Control of Midbrain Dopaminergic Neurons by Opioids

Elyssa Margolis

Abstract

Dopaminergic neurons of the ventral tegmental area (VTA) play a critical role in motivation and reinforcement of goal-directed behaviors. Excitation of these neurons has been implicated in the addictive process initiated by drugs such as morphine that act at the mu-opioid (MOP) receptor. In contrast, kappa-opioid (KOP) receptor activation in the VTA produces motivational actions opposite to those elicited by MOP receptor activation. The neural mechanism underlying this opposition, however, has not been investigated. VTA neurons have been categorized previously as principal, secondary, or tertiary on the basis of electrophysiological and pharmacological characteristics. In this thesis, post- and presynaptic actions of KOPs and MOPs in the VTA were investigated using whole-cell patch-clamp recordings.

In studies of the postsynaptic actions of opioids in VTA, I observed that a selective KOP receptor agonist (U69593, 1 μM) directly inhibits a subset of principal and tertiary, but not secondary, neurons. This KOP receptor-mediated inhibition occurs via activation of a G-protein-coupled inwardly rectifying potassium channel. Significantly, KOP receptor-mediated inhibition was limited to tyrosine hydroxylase immunoreactive, and thus dopaminergic, neurons. In addition, a subset of principal neurons was both disinhibited

by a selective MOP receptor agonist ([D-Ala 2, *N*-Me-Phe4, Gly-ol 5]-enkephalin) (3 μM) and directly inhibited by U69593.

Presynaptic MOP and KOP control of glutamate release onto the different classes of VTA neurons has not been systematically studied. To address this issue I measured excitatory postsynaptic currents (EPSCs) in VTA-containing brain slices. I confirmed that MOP agonists inhibit glutamate release onto principal and secondary cells, and discovered a similar effect in tertiary cells. I also found that U69593 produces a small reduction in EPSC amplitude in principal neurons, and a robust inhibition of EPSCs in secondary and tertiary neurons.

The postysynaptic results reported here provide a cellular mechanism for the opposing behavioral effects of KOP and MOP receptor agonists. In addition, the presynaptic actions of MOP and KOP agonists provide a mechanism for opioid control of specific inputs to each VTA cell class. Together, these data provide information essential to our understanding of how KOPs regulate the motivational effects of both natural rewards and addictive drugs.

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Chapter 1

Introduction

Neurons in the ventral tegmental area (VTA) are a major source of dopamine (DA) to structures in the forebrain and play a critical role in motivation and reinforcement. The firing of VTA dopaminergic neurons modulates general locomotor activity, behavioral switching, ingestive behavior, attack behavior, and self stimulation behavior (Koob, 1992). Furthermore, there is evidence that transient midbrain dopaminergic neuron firing provides an error signal related to reward expectancy that contributes to reinforcement (Schultz, 1998). VTA neurons are excited by natural rewards, such as food and sex (e.g. Giuliano and Allard, 2001), addictive drugs such as opiates (e.g. Cowen and Lawrence, 1999; Bonci et al., 2003) and by stressors (e.g. Feenstra, 2000; Moore et al., 2001; Saal et al., 2003). In view of the evidence linking forebrain dopamine with reinforcement and motivation, it is no surprise that scientists studying addiction have focused on the VTA and the actions of its efferents in target nuclei.

Many studies suggest that the positive reinforcement critical for the development of addictive behavior is mediated by a drug's ability to stimulate VTA dopaminergic neurons to release DA in their projection targets (Robinson and Berridge, 1993). When the signaling of the dopaminergic VTA system is altered by certain drugs, the changes can lead to addiction. Lesioning the dopaminergic system or blocking its function with antagonists prevents the formation and/or expression of many behavioral models of addiction (Phillips et al., 1983; e.g. Pettit et al., 1984; Di Chiara, 2000).

Opioids are of particular interest in this context because they can have powerful positive and negative motivational actions. There is strong evidence that the VTA is a critical site of opioid influence since behaviors elicited by systemic injections of drugs are very similar to those that result from intra-VTA injections of the same drugs. One widely accepted behavioral model for studying reinforcement in which this is the case is the conditioned place preference paradigm. In this paradigm, animals are injected with a drug and allowed to associate the effect of the drug with a room. Animals similarly associate control (saline) injections with a different room. Following this conditioning, animals are tested, with no drug on board, by giving them access to both rooms; then the amount of time spent in each room is analyzed. If the animal spends a greater amount of time in the drug-paired room following conditioning, it is said to have developed a conditioned place preference (CPP). If the animal avoids the drug-paired room, it has developed a conditioned place aversion (CPA). In this paradigm, μ opioid (MOP) receptor agonists elicit a conditioned place preference whether they are injected systemically (e.g. Iwamoto, 1985; Mucha and Herz, 1985) or directly into the VTA (Bals-Kubik et al., 1993). Similarly, κ opioid (KOP) receptor agonists elicit a conidditioned place *aversion* when given either systemically (e.g. Iwamoto, 1985; Mucha and Herz, 1985) or directly into the VTA (Bals-Kubik et al., 1993). Further, DA lesions or DA antagonists given systemically or in dopamine terminal regions block these behaviors (Shippenberg and Herz, 1987, 1988; McBride et al., 1999).

The VTA Neurons

The VTA is a midbrain structure most noted for its dopaminergic projection neurons. Two thirds of VTA neurons are dopaminergic and they comprise the A10 group of

dopaminergic neurons (Dahlstroem and Fuxe, 1964; Paxinos, 1995). The remaining 1/3 of VTA neurons mostly release the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Paxinos, 1995; Van Bockstaele and Pickel, 1995). In addition to providing local inhibition to dopamine neurons (Johnson and North, 1992b; Margolis et al., 2003), at least some of these GABA cells are projection neurons. While non-dopaminergic VTA neurons have a somewhat different projection pattern than dopaminergic neurons, many VTA target brain regions receive both types of input (Swanson, 1982; Van Bockstaele and Pickel, 1995).

VTA dopaminergic neurons can be divided into subpopulations. For example, some contain neurotensin, and both dopaminergic and non-dopaminergic neurons express cholecystokinin (Uhl et al., 1979; Hokfelt et al., 1980; Seroogy et al., 1989; Bayer et al., 1991a, b). There are also serotonergic neurons in the VTA (Jones and Cuello, 1989).

Despite this evidence that there are several subpopulations of VTA neurons, most electrophysiological attempts to identify neuron types have focused on delineating dopaminergic from non-dopaminergic neurons. Dopaminergic neurons in the VTA fire spontaneously at a low baseline firing rate of about 3 Hz, and their wide action potentials reach ½ peak amplitude in .92 msec (Johnson and North, 1992a). The action potential threshold for DA neurons is approximately -53 mV (Johnson and North, 1992a). Dopaminergic neurons recorded *in vivo* exhibit burst firing, and this bursting has been proposed to be a significant information-carrying signal for VTA output (Overton and Clark, 1997). *In vitro*, in whole cell recordings, immunohistochemically identified VTA dopaminergic neurons exhibit an I_h current: a non-selective cation current that can be observed as a current sag during hyperpolarizing voltage pulses (Johnson and North,

1992a). The I_h current is activated at or below -69 mV, grows as a single exponential, and exhibits no inactivation. It is hypothesized that this current prevents prolonged hyperpolarization of dopaminergic neurons (Mercuri et al., 1995).

One class of non-dopaminergic neurons in the VTA comprises GABAergic secondary cells. These neurons are morphologically, electrophysiologically, and pharmacologically distinct from the dopaminergic neurons. Secondary cells have much shorter action potentials (around 310 microsec), and fire at a much higher spontaneous rate (19 Hz) (Johnson and North, 1992a; Steffensen et al., 1998). Secondary cells do not burst fire, and, *in vitro*, they exhibit a more hyperpolarized resting membrane potential (-69 mV) and action potential threshold (-63 mV) than dopaminergic neurons (Johnson and North, 1992a). They also differ from other neuron types in the VTA in their lack of an I_h current, and their smaller, rounder shape. Early studies classified VTA neurons as either dopaminergic principal neurons or GABAergic secondary neurons.

In addition to dopaminergic and secondary neurons there is a third type in the VTA that is not dopaminergic but does have an I_h current (Fig. 1-1). These neurons are similar to dopaminergic neurons in most electrophysiological and morphological respects (see Chapter 4), however most appear to be non-dopaminergic (Cameron et al., 1997). Because electrophysiological characteristics therefore do not permit identification of dopaminergic neurons, immunohistochemical techniques are required. Whole cell recording methods allow a dye to be injected into the neuron during recording, and the tissue can be stained following the electrophysiological recording for tyrosine hydroxylase (TH), an enzyme that can be used to identify DA neurons. The tissue is then examined for the presence of TH in the dye-filled neuron. Using this approach, Jones

and Kauer (1999) found that 1/3 of neurons expressing a large I_h current were, in fact, not dopaminergic. Cameron, Wessendorf, and Williams (1997) also found a significant number of neurons that exhibited an I_h current but were non-dopaminergic. Importantly, a significant number of these non-dopaminergic neurons with an I_h were directly hyperpolarized by MOP receptor agonists. Cameron et al. therefore divided I_h-expressing neurons into principal neurons (80% dopaminergic) that are not inhibited by MOP receptor agonists, and tertiary neurons (40% dopaminergic) that are inhibited by MOP receptor agonists. In their study, tertiary neurons were relatively large with an average cross-sectional area of 781±118 μm^2, as compared to 352±52 μm^2 for principal neurons, and were either fusiform or pyramidal in shape (Cameron et al., 1997). While distinguishing between principal and tertiary neurons is useful, it is clearly a flawed tool for identifying dopamine neurons; there are at least four distinct types of I_h expressing neurons: dopaminergic (with and without a MOP hyperpolarization) and non-dopaminergic (with or without a MOP hyperpolarization).

VTA Anatomy

Neurons in the VTA project primarily to one of 3 major brain regions. Axons of VTA dopaminergic neurons are a principal component of the medial forebrain bundle (MFB), which, among other things, connects the VTA to the ventral striatum, olfactory bulb, amygdala (AMYG; central, medial, and lateral), anterior limbic neocortex , entorhinal area, and the hypothalamus (Fig. 1-2, Swanson, 1982). The projection to the NAc is over 80% dopaminergic and probably arises from throughout the VTA (Swanson, 1982), although there is some evidence for a more limited projection from the medial-dorsal region (Paxinos, 1995). The projection to the medial prefrontal cortex (mPFC) is

less than 40% dopaminergic, and there is similar controversy about whether the projection arises diffusely from throughout the VTA (Swanson, 1982) or from a more limited region that is medial (Lindvall et al., 1978; Simon et al., 1979) or ventral (Beckstead, 1976). The AMYG projection arises from the ventral 2/3 of the VTA, and is 50% dopaminergic (Swanson, 1982). A second projection is caudally directed to the periaqueductal gray, parabrachial nucleus, locus coeruleus, and median raphe nucleus, and at least in the case of the locus coeruleus, is mostly non-dopaminergic (Swanson, 1982). Notably, the locus coeruleus and parabrachial nucleus are the only identified brain regions that receive a highly crossed (over 40%) projection from the VTA (Beckstead et al., 1979; Swanson, 1982). The third major VTA projection terminates in the thalamus (dorsal to medial), including the lateral habenula (Swanson, 1982). In retrograde double-labeling experiments, virtually no neurons were found to project to more than one brain region (Swanson, 1982).

Inputs to the VTA arise from throughout the limbic system. Excitatory fibers descend from the limbic cortex and the hypothalamus through the MFB (Phillipson, 1979; Swanson, 1982). The mPFC projections include fibers from the infralimbic area of Rose and Woolsey and the prelimbic area of Kretek and Price (Phillipson, 1979). Another large projection comes from the dorsal bank of the rhinal sulcus including the ventral agranular insular area and the ventrolateral orbital area (Phillipson, 1979). Hypothalamic input to the VTA consists of a ventromedial projection to the medial VTA (Paxinos, 1995), and a diffuse projection from the lateral hypothalamus (Phillipson, 1979). The VTA also receives glutamatergic input from the hippocampus, pedunculopontine nucleus, and subthalamic nucleus (Paxinos, 1995).

Other brain regions supply GABAergic, serotonergic, and noradrenergic inputs to the VTA. The NAc and ventral pallidum (VP) send GABAergic projections to the VTA. Frontal and cingulate cortical projections include GABA in addition to glutamate (Phillipson, 1979; Paxinos, 1995). Locus coeruleus projections release noradrenalin in the VTA (Phillipson, 1979). A dense projection from the dorsal raphe probably supplies the VTA with serotonin (Phillipson, 1979). Less studied projections to the VTA include the lateral habenula, deeper layers of the superior colliculus and lateral and medial portions of the preoptic area (Phillipson, 1979; Paxinos, 1995).

Although there is no strong topographical organization to the VTA, some more specific circuit connections have been determined. Within the VTA, there is tonic inhibitory control of dopaminergic neurons, most likely from intrinsic VTA GABAergic interneurons (presumably secondary cells) or collaterals of GABAergic projection neurons (Margolis et al., 2003). It is important to note that dopamine also inhibits dopaminergic neurons directly and facilitates stimulated GABA-B inhibitory postsynaptic potentials (IPSPs) *in vitro* (Johnson and North, 1992a; Cameron and Williams, 1993). Measurable extracellular dopamine is present in the VTA, although whether it is released from dendrites or local collaterals is not entirely clear (e.g. Bradberry and Roth, 1989; Klitenick et al., 1992; Yoshida et al., 1992).

Carr and Sesack (2000) have shown a very specific VTA afferent-efferent pattern for the mPFC and NAc. Projections from the mPFC to the VTA synapse only onto those dopaminergic neurons that project back to the mPFC. Also, non-dopaminergic neurons that receive input from the mPFC project to the NAc, but not the mPFC (Fig. 1-3). This

suggests that efferent and afferent connections have a distinct organization even though the VTA does not have a clear topography.

Opioids

Both endogenous and exogenous opioid ligands modulate motivational states through actions in the VTA. Therefore investigating the anatomical and functional properties of the different opioid systems within the VTA will lead to insights about the VTA's contribution to reinforcement and motivation. Furthermore, such knowledge will advance our understanding of the mechanisms responsible for the development and maintenance of addiction.

The opioid receptors (MOP, delta (DOP), KOP, and opioid receptor like (ORL1)) and the endogenous opioid ligands (endomorphin (EM), enkephalin (ENK), dynorphin (DYN), and nociceptin/orphanin FQ (N/OFQ)) all have powerful influences on motivation. MOP receptors are of particular interest because they are directly activated by abused opiate drugs. Furthermore, there is a growing body of evidence that the endogenous opioid systems contribute to the addictive power of non-opiate drugs such as cocaine and ethanol (Nylander et al., 1995; Shippenberg and Rea, 1997; Rosin et al., 1999; Magendzo and Bustos, 2003). The VTA is a logical place to study the interaction of the various opioid receptor classes since all are present in this structure and it exerts widespread control over the forebrain. The importance of the VTA to addiction is indicated by the observation that behaviors elicited in response to opioids delivered directly to the VTA are generally the same as those elicited by systemic drug administration. Because of the receptor selectivity of opioid drugs and our ability to pinpoint the behaviorally relevant receptor locations and cellular responses to receptor

agonists, the circuits modulated by opioids provide prime targets for the development of medications to treat drug addiction.

Mu Opioid System in the VTA

Anatomy and Function

The MOP receptor is by far the most thoroughly studied opioid subtype in the VTA from anatomy to behavior. Anatomical studies not only locate MOP receptors and endogenous ligands in the VTA, but give some insight into both intra-VTA circuitry and VTA connections with other brain regions. MOP receptors have been immunocytochemically localized primarily in non-dopaminergic neurons (Garzon and Pickel, 2001). Many of these neurons likely correspond to those that are directly inhibited by MOP receptor agonists through the activation of a somadendritic G-protein coupled inwardly rectifying potassium channel (Johnson and North, 1992a, b; Cameron et al., 1997). There is anatomical evidence that some of these neurons project to the mPFC (Svingos et al., 2001).

Postsynaptic MOP receptors have also been found on neurons, possibly local GABAergic interneurons, that synapse onto other VTA neurons (Svingos et al., 2001). These are the neurons whose inhibition by MOP agonists has been proposed to disinhibit dopaminergic neurons (Fig. 1-4, Margolis et al., 2003). Morphine, which activates both MOP and DOP receptors, inhibits secondary neurons and excites putative dopamine neurons *in vivo* (Gysling and Wang, 1983; Kiyatkin, 1988). This disinhibition likely underlies increases in both dopamine and dopamine metabolite concentrations measured by microdialysis in the NAc following intra-VTA administration of MOP receptor agonists, such as DAMGO and morphine (Devine et al., 1993b; Yoshida et al., 1993).

Increases in dopamine concentration have also been observed within the VTA following reverse-dialysis of MOP receptor agonists into the VTA (Devine et al., 1993b). Excitation of dopaminergic neurons through disinhibition is thought to be one of the critical mechanisms by which drugs with MOP receptor agonist efficacy activate motivational circuits associated with addiction.

The interpretation of dopamine microdialysis studies in which MOP receptor antagonists are infused into the VTA is not as straightforward. Administering a MOP receptor antagonist alone into the VTA increases dopamine levels in the NAc (Devine et al., 1993b; Devine et al., 1993a). These data suggest that there is not only tonic MOP receptor activation in the VTA, but that this endogenous opioid tone works to somehow block the excitation of dopaminergic neurons under basal conditions. This is very different from the model of opioid activation disinhibiting DA neurons. A candidate mechanism for this is the presynaptic inhibition of glutamate release onto dopaminergic neurons (Bonci and Malenka, 1999; Manzoni and Williams, 1999). Conversely, others have reported that MOP receptor antagonist infusion into the VTA produces a decrease in NAc dopamine and blocks the increases in NAc dopamine that are observed following systemic nicotine administration and palatable food consumption (Spanagel et al., 1992; Tanda and Di Chiara, 1998). These results are more in line with the predominant view that endogenous MOP receptor ligands play a positive role in the development of addiction by exciting VTA dopaminergic neurons.

MOP receptors have also been identified on presynaptic axons in the VTA and about half of these synapse onto dopaminergic neurons (Garzon and Pickel, 2001). Corresponding presynaptic effects on transmitter release by MOP agonists have also been

demonstrated. MOP receptor agonists presynaptically inhibit excitatory postsynaptic currents (EPSCs) in principal and secondary neurons (tertiary neurons were not studied) (Bonci and Malenka, 1999; Manzoni and Williams, 1999). MOP agonists also inhibit GABA-A receptor mediated IPSPs in neurons with an I_h (principal and tertiary neurons were not separated in this study), but have not been studied in secondary neurons (Bonci and Williams, 1997). Despite the evidence for both pre- and postsynaptic opioid actions, no attempts have been made to date to understand the relationship between these two types of MOP effect in the VTA. Such information might resolve the conflicting observations following VTA injections of MOP receptor antagonists.

Because MOP receptor expression in the VTA changes following exposure to drugs of abuse, it is likely that the relationship between these different MOP receptor regulated responses also changes during the development of addiction. For example, MOP receptor expression in the VTA decreases 30 minutes after ethanol administration (Mendez et al., 2001). On the other hand, animals that are sensitized to amphetamine show an increase in MOP receptor expression in the VTA two days after the drug administration. This elevated receptor expression persists for at least 14 days (Magendzo and Bustos, 2003).

Changes concomitant with morphine withdrawal have also been demonstrated electrophysiologically. Neurons in morphine withdrawn slices exhibit more spontaneous miniature IPSPs, and a decrease in the paired pulse ratio, both of which suggest an increased probability of neurotransmitter release (Bonci and Williams, 1997). Evoked GABA-A IPSPs are also larger overall in brain slices from morphine withdrawn animals, additional evidence that long-term exposure to MOP receptor agonists alters the functioning of the GABAergic system within the VTA (Manzoni and Williams, 1999).

The MOP receptor binds both endogenous opioid ligands and morphine. EM-1 and -2, which selectively bind to the MOP receptor, are present in the VTA at modest levels in afferents from the hypothalamus (Greenwell et al., 2002). ENK, which is co-contained in GABAergic projections from the VP to the VTA, activates both the MOP and DOP receptors. These enkephalinergic projections synapse onto DA neurons (Sesack and Pickel, 1992). Opiate withdrawal increases the ENK levels in the VTA (Nylander et al., 1995). Blocking the action of these endogenous MOP ligands is a potential mechanism by which general opioid antagonists, such as naloxone, work as alcoholism therapeutics.

Behavior

Behavioral responses to microinjections of MOP receptor agonists into the VTA have been measured in a variety of experimental paradigms. All of the behavioral findings mentioned below are from direct VTA microinjection studies.

One basic behavioral effect of MOP receptor activation in the VTA is increased arousal, as measured by locomotor activity. MOP agonists administered directly into the VTA increase locomotion (Kelley et al., 1980; Latimer et al., 1987; Calenco-Choukroun et al., 1991; Klitenick and Wirtshafter, 1995; Zangen et al., 2002) and this behavioral effect can be blocked by dopamine-selective lesions of certain VTA terminal fields (Kelley et al., 1980). This 'arousal' effect seems to be more potent in the caudal VTA than the rostral VTA, at least in the case of EM-1 (Zangen et al., 2002). Unilateral injections of MOP receptor agonists in the VTA lead to circling, and this effect is blocked by systemic haloperidol, a dopamine antagonist, suggesting opioids affect locomotor behaviors by modulating dopamine neurons (Szewczak and Spoerlein, 1986; Jenck et al., 1988).

MOPs in the VTA produce behavioral sensitization (Kelley et al., 1980; Kalivas et al., 1985; Vezina et al., 1987) and modify the sensitizing effects of other drugs. In the addiction literature, behavioral sensitization specifically refers to the progressive increases in locomotor activity produced by successive administration of the same dose of a drug. Sensitization to drugs of abuse may reflect a process that contributes to the development of addiction (Kalivas et al., 1998; Cornish and Kalivas, 2001; Schenk et al., 2001). MOP agonists induce sensitization that is not only expressed in subsequent administrations of MOP receptor agonists, but MOPs also cross-sensitize with systemically administered amphetamine (Kelley et al., 1980; Chen et al., 2001).

Caloric and water intake are other basic behaviors that are modulated by opioids in the VTA. Specifically, MOP receptor selective agonists administered directly into the VTA increase food consumption (Mucha and Herz, 1985; Hamilton and Bozarth, 1988; Lamonte et al., 2002). Breaking down the behavior further has not led to consistent observations: one study showed that MOP receptor agonists decrease the latency to feeding and increase the number of interactions with food (Badiani et al., 1995b), while another showed an increase in feeding speed but no change in latency to obtaining food (Noel and Wise, 1995). While MOP receptor selective antagonists in the VTA have no effect on feeding (Badiani et al., 1995a), the non-specific opioid antagonist naltrexone decreases food intake (Ragnauth et al., 1997), suggesting that although feeding can be enhanced by MOP agonists, it normally requires non-MOP activation of the VTA.

Behavioral studies investigating MOP receptor agonist microinjections suggest that opioid function in the VTA is sufficient to produce a variety of reward- and addiction-related behaviors. One such behavioral model is CPP, as described above. MOP

receptor agonists produce CPP when administered systemically as well as directly into the VTA (Phillips and LePiane, 1980; Bals-Kubik et al., 1993; Nader and van der Kooy, 1997). This behavior may have some topography in the VTA: EM-1 microinjected into the caudal VTA results in robust CPP, while rostral VTA injections do not (Zangen et al., 2002). However, similar studies with morphine have not reported this difference (Bozarth, 1987; Carlezon et al., 2000), possibly due to morphine activating DOP receptors in addition to MOP receptors. Interestingly, if the VTA is transfected with a gene to increase the expression of the AMPA glutamate receptor subunit GluR1, a topography to morphine CPP develops, and rostral VTA injections of morphine produce CPP while caudal morphine injections produce the affective opposite, CPA (Carlezon et al., 2000). The MOP receptor antagonist CTOP in the VTA will produce CPA (Shippenberg and Bals-Kubik, 1995), suggesting some basal endogenous VTA MOP receptor activation.

It is important to note that although these studies show that modulating VTA output is sufficient for the induction of place conditioning, Nader and van der Kooy (1997) showed that dopamine is only necessary for establishing CPP in opiate dependent and withdrawn rats, not those that were drug naïve preceding CPP training. However, dopaminergic lesions or GABA-B agonist injections into the VTA block the reinstatement of morphine conditioned place preference following a priming dose of morphine (Tsuji et al., 1996; Wang et al., 2003). Further, the fact that MOP receptor agonist injections into the NAc, mPFC and lateral hypothalamus do not elicit CPP underscores the relative importance of MOP receptors in the VTA for morphine addiction (Bals-Kubik et al., 1993; Zangen et al., 2002).

Self administration of a drug is another common behavioral paradigm, and is often thought of as an animal model of addiction. Rats can be trained to recognize that the delivery of a reward, such as a drug, is contingent upon their performance of a task, such as lever-pressing and nose-poking. Rats will lever press for MOP receptor agonist delivery directly into the VTA (Devine and Wise, 1994; Zangen et al., 2002).

MOP receptor activation in the VTA also modifies responding during self-administration tasks for other "rewards." Intra-VTA injection of the MOP receptor agonist DAMGO will increase self administration of cocaine as well as intracranial self-stimulation (ICSS) in the hypothalamus (Singh et al., 1994; Corrigall et al., 1999). ENK and DAMGO injections into the VTA increase responding for food pellets (Kelley et al., 1989; Corrigall et al., 2000). In rats trained to self administer drugs and consequently extinguished by replacing the drug injections with saline injections, morphine reinstates seeking and self administration of heroin and cocaine, but not nicotine (Stewart, 1984; Corrigall et al., 2000).

In addition to food consumption, place preference and drug self-administration, VTA MOP receptor activation modulates a variety of other basic behaviors. Sexual behaviors in male rats, measured as female-directed behaviors, number of ejaculations, and copulation latencies, are increased by morphine injected into the VTA or by environmental cues associated with previous VTA morphine injections (Mitchell and Stewart, 1990a, b). The ENK analog DAME decreases and naloxone increases predatory behavior elicited through electrical stimulation of the hypothalamus (Bhatia et al., 1997). It is important to consider these behaviors that are influenced by the VTA, and modulated

by opioid receptors there, because they may provide broader insight into the general role played by the VTA in motivation and behavioral choice.

Taken together, these behavioral studies suggest that the endogenous MOP system in the VTA influences, and is likely critical for, instinctive behaviors like feeding and aggression, and also the learned goal directed behaviors used to investigate addiction. The extensive knowledge we have of MOP effects in the VTA make it an excellent model system to use to investigate how changes in synaptic transmission in the VTA influence motivation and reinforcement.

Delta Opioid System in the VTA

Anatomy and Function

The DOP system in the VTA is not as thoroughly studied as the MOP system. While DOP receptors have been localized in the VTA, their immunohistochemically determined expression level is much lower than that for MOP receptors (Bausch et al., 1995; Cahill et al., 2001). ENK projections from the VP and NAc activate DOP receptors as well as MOP receptors (Kalivas et al., 1993). Morphine also activates both MOP and DOP receptors. DOP system activation seems to produce behavioral outputs similar to those produced by the MOP system in the VTA. However, much higher concentrations of highly selective and efficacious DOP agonists are usually required to observe equivalent physiological and behavioral changes. Similar to MOP receptor agonists, DOP receptor agonists in the VTA increase dopamine concentration in the NAc measured by microdialysis; however the amount of agonist required to produce equivalent increases in DA levels is two orders of magnitude larger than for MOP receptor agonists (Devine et

al., 1993c; Devine et al., 1993b). Conversely, DOP receptor antagonists in the VTA decrease dopamine concentrations in the NAc (Devine et al., 1993b).

There are some interesting differences in the dynamic regulation of MOP and DOP receptor systems. For instance, animals that are sensitized to amphetamine show a transient increase in DOP receptor expression in the VTA at two, but not 14, days after the drug administration (Magendzo and Bustos, 2003). This temporary increase is very different from the prolonged increase in MOP receptor expression that is elevated for at least 14 days.

Behavior

Behavioral effects of VTA microinjections of DOP agonists are also similar to those elicited by MOP receptor agonists, but again, much larger doses are required in order to obtain responses of a similar magnitude. DOP receptor agonists such as ENK and DPDPE stimulate locomotor behavior (Joyce et al., 1981; Latimer et al., 1987; Calenco-Choukroun et al., 1991). Systemic and intra-VTA injections of DOP receptor antagonists block this locomotor behavior enhancement (Calenco-Choukroun et al., 1991). Interestingly, intra-VTA DOP receptor selective agonist induced locomotor behavior is also blocked by naloxone, but not by the dopamine antagonist alpha-flupentixol (Joyce et al., 1981). As with MOP, unilateral injections of DOP receptor agonists in the VTA induce circling that is reversed with systemic haloperidol (Szewczak and Spoerlein, 1986; Jenck et al., 1988). A DOP receptor selective antagonist will decrease feeding when administered to the VTA (Ragnauth et al., 1997; Lamonte et al., 2002). ENK, acting at both MOP and DOP receptors, decreases predatory behavior (Bhatia et al., 1997).

Operant responding is also modulated by DOP receptors in the VTA. Rats will lever press for DOP receptor agonist delivery directly into the VTA (Devine and Wise, 1994; Zangen et al., 2002). As in the microdialysis studies mentioned above, the dose required to maintain this behavior is 100 times greater than that for MOP receptor agonist self administration. DOP receptor agonists in the VTA also increase ICSS in the hypothalamus (Singh and Desiraju, 1988; Singh et al., 1994). ENK injections into the VTA increase operant behaving for food pellets (Kelley et al., 1989), and it seems likely that ENK exerts this effect through the activation of both the MOP and DOP receptors.

Together, these results are consistent with the view that the endogenous MOP and DOP systems in the VTA act cooperatively to influence motivation. Although DOP agonists produce effects very similar to those produced by MOPs in the VTA, there still are questions raised by comparing these two opioid receptor systems. In order to understand the MOP-DOP relationship in the intact system, it is important to know if endogenously released ENK activates one or both types of receptors. Whether or not the intact system is organized such that MOP and DOP receptors are activated concomitantly by ENK, and the differential influences of endogenously released EM and ENK, may prove important for understanding the contribution of endogenous opioids to the actions of drugs like ethanol and morphine that elicit their release.

Kappa Opioid System in the VTA

Anatomy and Function

The KOP system in the VTA is of great interest because it can produce behavioral responses that are powerful and opposite to those activated by MOP and DOP agonists. Anatomically, KOP receptors have been immunohistochemically and

autoradiographically identified in the VTA (Speciale et al., 1993; Arvidsson et al., 1995; Mansour et al., 1996). KOP receptor activation in the NAc decreases local DA release, suggesting that KOP receptors are also present on the dopamine terminals in the NAc (Spanagel et al., 1990). Dynorphin, the endogenous KOP ligand (Chavkin et al., 1982), is released in the VTA from the terminals of neurons located in a number of sites. It is colocalized with GABA and substance P in fibers that arise from the NAc (Paxinos, 1995). It is also colocalized with orexin and probably glutamate in terminals arising from neurons in the lateral hypothalamus (Chou et al., 2001; Rosin et al., 2003). There is also a small projection from the AMYG that contains DYN (Fallon et al., 1985). The majority of terminals containing DYN appose dopaminergic dendrites, and most synapses are symmetric, an indication that they are inhibitory (Pickel et al., 1993).

There is evidence that the KOP system in the VTA can modulate the development of addiction, even though drugs of abuse do not directly activate KOP receptors. Ethanol-preferring rats have lower baseline DYN levels than non-drinking rats (Nylander et al., 1994). Exposure to drugs of abuse changes the expression levels of the DYN/KOP system in a variety of brain regions. For example, morphine treatment increases DYN levels in the VTA (Nylander et al., 1995). Furthermore, KOP receptor mRNA expression is decreased to about 10% of baseline levels in the VTA following 14 days of binge cocaine or alcohol exposure, and the reduction is greater following concurrent administration of both drugs (Rosin et al., 1999).

Despite their demonstrated importance in regulating motivated behaviors, prior to the studies included in this thesis, little was known of the synaptic actions of KOP receptor agonists in the VTA. Furthermore, in spite of studies showing a decrease in dopamine

release in the NAc following systemic injections of KOP receptor agonists, microinjections of KOP receptor agonists into the VTA of anesthetized rats failed to produce a decrease in dopamine concentrations in the NAc (Spanagel et al., 1992; Devine et al., 1993a). However, microinjections of KOP receptor agonists in the VTA decrease extracellular dopamine in the NAc after it has been increased by systemic administration of haloperidol (Leyton et al., 1992). At the synaptic level, Manzoni and Williams (1999) reported that there was no presynaptic effect of KOP receptor agonists on glutamatergic EPSCs recorded in VTA principal neurons. This leaves open the major issue addressed in this thesis: what are the synaptic consequences of activating KOP receptors in the VTA?

Behavior

KOP injections into the VTA tend to elicit very different behaviors from MOP and DOP receptor agonists. Unlike MOP and DOP receptor agonists, unilateral injections of KOP receptor agonists in the VTA do not lead to circling (Szewczak and Spoerlein, 1986; Jenck et al., 1988), nor do they increase locomotor activity (Leyton and Stewart, 1992; Klitenick and Wirtshafter, 1995). In fact, DYN injections into the VTA block the increased locomotion induced by ENK injections into the VTA (Kalivas et al., 1985). In contrast to the place preference produced by MOP agonists, KOP receptor agonists produce place *aversion* when injected in the VTA (Bals-Kubik et al., 1993). And, instead of cross-sensitizing with cocaine like MOPs, KOP receptor agonists given systemically *block* behavioral sensitization to cocaine (Shippenberg and Rea, 1997).

There is evidence in other brain regions that KOP receptor activation has differing effects on motivation, depending on the state of the animal (Bie and Pan, 2003). This

heterogeneity may also apply in the VTA, as there are some behaviors in which KOP receptor agonists in the VTA do not oppose the actions of MOP and DOP. For instance, DYN injected into the VTA stimulates feeding (Hamilton and Bozarth, 1988), and a KOP receptor selective *antagonist* reduces MOP-elicited feeding (Lamonte et al., 2002). KOP receptor agonists, like MOP and DOP, in the VTA also increase ICSS in the hypothalamus. KOP receptor agonists are the most effective opioid in this paradigm, increasing responding by over 50% (Singh and Desiraju, 1988; Singh et al., 1994).

Taken together, these observations suggest a complex interaction between the KOP and MOP systems in the VTA. While some behavioral tests, such as CPP, clearly show opposing actions of MOP and KOP in the VTA, the feeding and ICSS results suggest that these systems have the potential to act in concert in the VTA. How can we reconcile these seemingly paradoxical findings from behavioral pharmacology experiments?

One possibility of course is that distinct subpopulations of VTA neurons will be differentially sensitive to the direct postsynaptic actions of MOP and/or KOP receptor agonists, and these subpopulations will be further defined by their anatomical connectivity. For instance, KOP and MOP receptor activation could similarly affect VTA output that influences feeding behaviors while having opposing effects on VTA output mediating place conditioning. Another possibility is that MOP, DOP and KOP effects are primarily presynaptic and act on distinct sets of afferent terminals that are activated under different behavioral conditions. Clearly, distilling out the similarities and differences between the influences of MOP, DOP and KOP systems in the VTA that lead to the behavioral observations reviewed here will require detailed knowledge of both the pre- and postsynaptic actions exerted by ligands for each receptor type.

Orphanin System in the VTA

Anatomy and Function

The ORL1 system is the most recently discovered member of the opioid family, and both the ORL1 receptor and endogenous ligand N/OFQ are expressed in the VTA (Neal et al., 1999b; Neal et al., 1999a). Anatomical studies have shown that ORL1 receptor mRNA is located mostly in dopaminergic neurons, and most dopaminergic neurons in the VTA express ORL1 receptor mRNA (Maidment et al., 2002; Norton et al., 2002). N/OFQ has been localized in the VTA both through immunohistochemistry and in situ hybridization for prepro-N/OFQ (Neal et al., 1999a). Additionally, mRNA for N/OFQ is present in a subpopulation of mostly GABAergic VTA neurons (Norton et al., 2002).

The N/OFQ system, like the MOP system, seems to modulate VTA function through a number of mechanisms. Orphanin postsynaptically hyperpolarizes a subset of both dopaminergic and non-dopaminergic neurons (Zheng et al., 2002). As expected from this *in vitro* observation, microinjections of N/OFQ into the VTA decrease dopamine release in the NAc (Murphy and Maidment, 1999). Orphanin also inhibits spontaneous GABA-A IPSPs in principal neurons *in vitro* in brain slices (Zheng et al., 2002). This includes TTX-sensitive spontaneous IPSPs, suggesting that neurons that are spontaneously active in the VTA slice and are inhibited by N/OFQ make inhibitory synapses onto neighboring neurons (Zheng et al., 2002). This hypothesis is supported by microdialysis data showing that reverse dialysis of orphanin into the VTA decreases local GABA concentrations (Murphy and Maidment, 1999). Extracellular glutamate in the VTA is also decreased in these conditions, although a specific mechanism to account for this observation has not yet been determined (Murphy and Maidment, 1999)

Behavior

Few studies have been completed investigating the behavioral effects of ORL1 receptor activation in the VTA. Systemically, N/OFQ does not produce a conditioned place preference or aversion (Devine et al., 1996). However, since N/OFQ inhibits dopaminergic neurons and decreases extracellular dopamine in the NAc, one may hypothesize that N/OFQ locally injected into the VTA will induce CPA. If so, activating the KOP and ORL1 systems in the VTA would have similar place conditioning effects.

The ORL1 system's regulation of cocaine sensitization in the VTA has been investigated. Specifically, microinjections of an ORL1 agonist into the VTA sensitizes subsequent locomotor responses to cocaine a day later (Narayanan et al., 2002). However, if N/OFQ is injected into the VTA 5 minutes prior to cocaine administration, animals show no locomotor response to the cocaine (Lutfy et al., 2002; Narayanan et al., 2002). Activation of the KOP system also blocks sensitization (Chefer et al., 2000). Yet this result was observed following systemic administration of KOP receptor agonists, thus leaving open the possibility that KOPs block sensitization through a non-VTA mechanism.

Although N/OFQ has been shown to modulate synaptic transmission in the VTA through more than one mechanism, little is currently known about the behavioral consequences of these effects. It would be particularly interesting to know if intra-VTA injections of N/OFQ lack motivational effects in the same way that systemic injections do. Clearly there is still much to learn of the role played by the ORL1 system in the VTA.

Experimental Rationale

The findings summarized above establish the VTA as an important site of opioid action. Thus, determining how each opioid receptor modulates each type of VTA neuron is critical for understanding how these neurons contribute to motivation. The most extensive behavioral data specific to the VTA exists for MOP and KOP receptor systems, including evidence that these systems can produce opposing motivational effects through their direct actions in the VTA. Since the known pre- and postsynaptic actions of MOP and KOP receptor activation are similar, it follows that the behavioral differences are due to a segregation of the two receptor classes on to different neural elements in the VTA. Yet, no synaptic actions of KOP receptor agonists in the VTA had been reported prior to this thesis.

There is a body of evidence suggesting that the source of the opposing effects of MOPs and KOPs is their differential modulation of dopamine neurons in the VTA. The stimulation of DA release by certain drugs is hypothesized to be one of the key mechanisms responsible for their addictive quality. Therefore, if KOP receptor agonists indeed inhibit dopaminergic neurons, it is possible that an appropriately designed therapeutic KOP drug could functionally oppose the stimulation of dopamine neurons elicited by addictive drugs and therefore decrease their reinforcing quality.

The experiments presented in this thesis investigate the signal modulation properties of KOP receptor agonists in the VTA, and compare them to those of MOP receptor agonists. The first set of experiments examines the postsynaptic responses of all neuron types in the VTA to both KOP and MOP agonists. The second group of experiments was designed to determine the presynaptic effects of KOP and MOP receptor agonists on

glutamate release in the VTA through observation of AMPA-mediated excitatory postsynaptic currents. The last set of data presented here is an examination of a variety of anatomical, pharmacological, electrophysiological, and firing pattern properties of I_h neurons in an attempt to discern a reliable *in vitro* marker that predicts dopamine content. Overall, this study will not only inform rational drug design, but it will add to our growing understanding of the reward system and how disruptions in this system lead to addiction.

Figure Legends

Figure 1-1. The absence of an I_h current delineates one subpopulation of non-dopaminergic neurons in the Ventral Tegmental Area (VTA).

Figure 1-2. The VTA is connected to forebrain structures through the medial forebrain bundle.

Figure 1-3. The medial prefrontal cortex preferentially projects to VTA dopaminergic neurons that project back to the cortex. In contrast, VTA GABAergic neurons that receive input from the mPFC project to the NAc, but not back to the mPFC.

Figure 1-4. Mu opioid(MOP) receptor agonists indirectly activate dopaminergic (DA) neurons in the VTA. GABAergic neurons in the VTA that provide tonic inhibition onto DA neurons are inhibited by MOP receptor agonists, thereby disinhibiting dopaminergic neurons.

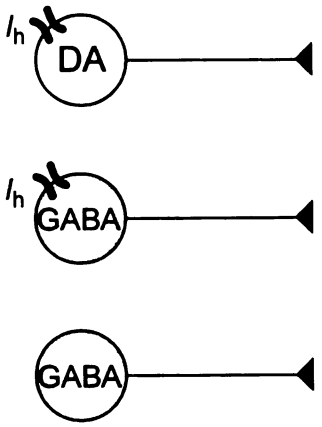

Figure 1-1

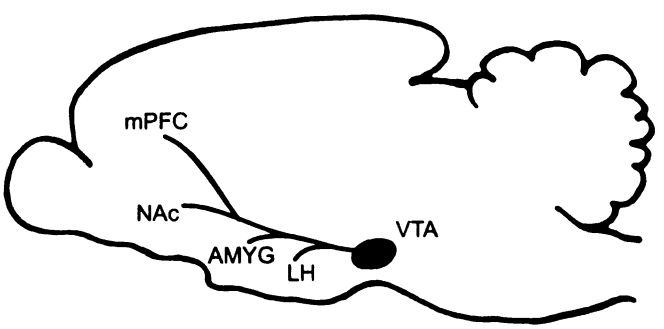

Figure 1-2

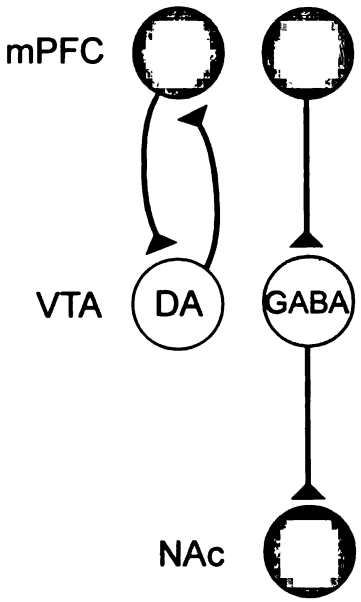

Figure 1-3

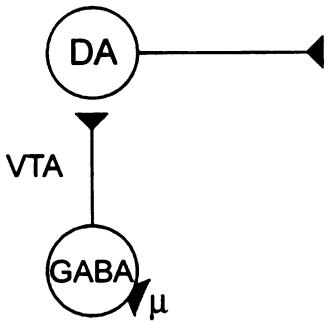

Figure 1-4

Chapter 2

Kappa Opioid Agonists Directly Inhibit Midbrain Dopaminergic Neurons

Elyssa B. Margolis[1], Gregory O. Hjelmstad[2], Antonello Bonci[2], & Howard L. Fields[1,2]

1. Joint UCSF/UCB Bioengineering Graduate Group, San Francisco, CA 94143-0775

2. Dept. of Neurology, Wheeler Center for the Neurobiology of Addiction, Ernest Gallo Clinic & Research Center, University of California, San Francisco, California 94143-0114.

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Abstract

Dopaminergic neurons of the ventral tegmental area (VTA) play a critical role in motivation and reinforcement of goal-directed behaviors. Furthermore, excitation of these neurons has been implicated in the addictive process initiated by drugs such as morphine that act at the micro-opioid receptor (MOR). In contrast, kappa-opioid receptor (KOR) activation in the VTA produces behavioral actions opposite to those elicited by MOR activation. The mechanism underlying this functional opposition, however, is poorly understood. VTA neurons have been categorized previously as principal, secondary, or tertiary on the basis of electrophysiological and pharmacological characteristics. In the present study using whole-cell patch-clamp recordings, we demonstrate that a selective

KOR agonist (U69593, 1 μM) directly inhibits a subset of principal and tertiary but not secondary neurons in the VTA. This KOR-mediated inhibition occurs via the activation of a G-protein-coupled inwardly rectifying potassium channel and is blocked by the selective KOR antagonist nor-Binaltorphimine (100 nm). Significantly, regardless of cell class, KOR-mediated inhibition was found only in tyrosine hydroxylase-immunoreactive and thus dopaminergic neurons. In addition, we found a subset of principal neurons that exhibited both disinhibition by a selective MOR agonist ([d-Ala2, N-Me-Phe4, Gly-ol5]-enkephalin) (3 μM) and direct inhibition by KOR agonists. These results provide a cellular mechanism for the opposing behavioral effects of KOR and MOR agonists and shed light on how KORs might regulate the motivational effects of both natural rewards and addictive drugs.

Introduction

Kappa and mu opioid receptor (KOR and MOR) agonists have opposing actions in a number of systems in the brain, including those mediating reward, motivation, and pain modulation (Pan, 1998). The ventral tegmental area (VTA) is a critical site for opioid effects on motivation. Intra-VTA injections of MOR agonists elicit conditioned place preference (CPP): rats spend more time in a context paired with MOR agonist administration than in a saline-paired environment (Phillips and LePiane, 1980; Bals-Kubik et al., 1993; Nader and van der Kooy, 1997). Conversely, intra-VTA microinjections of KOR agonists produce conditioned place aversion (CPA) (Bals-Kubik et al., 1993).

There is evidence that an increase in dopamine (DA) release from VTA neurons contributes to the positive motivational actions of MOR agonists. VTA dopaminergic neurons project densely to the nucleus accumbens (NAc), and dialysis studies show that VTA microinjections of the MOR selective agonist DAMGO increase DA release in the NAc (Latimer et al., 1987; Leone et al., 1991; Spanagel et al., 1992; Devine et al., 1993a). Furthermore, a DA action in the NAc is critical for MOR agonist-elicited behavioral effects. In opiate-exposed rats, CPP produced by intra-VTA morphine is blocked by systemic dopamine antagonists (Nader and van der Kooy, 1997), and MOR agonist induced CPP is blocked by 6-OHDA destruction of NAc DA terminals (Shippenberg et al., 1993). The requirement for DA in expression of morphine CPP supports the hypothesis that enhanced firing of VTA dopaminergic neurons contributes to the positive motivational actions of MOR agonists.

Do KOR agonists contribute to aversion by *decreasing* the firing rate of VTA dopaminergic neurons? KORs are functionally expressed in the VTA (Speciale et al., 1993; Arvidsson et al., 1995; Mansour et al., 1996) and dynorphin, an endogenous opioid peptide with high selectivity for the KOR, is present in projections to the VTA from several structures strongly implicated in motivation and reinforcement, including the NAc, amygdala and hypothalamus (Fallon et al., 1985; Meredith, 1999). Although KOR agonists inhibit DA release from cultured VTA neurons (Ronken et al., 1993; Dalman and O'Malley, 1999), this decrease in DA release does not distinguish an effect at the cell body from one on the terminals of the cultured neuron. Because microinjections of KOR agonists directly into the NAc *in vivo* produce place aversions and inhibit DA release (Bals-Kubik et al., 1993), several investigators have proposed that the aversive action of

systemically administered KOR agonists is mediated primarily by their direct inhibition of DA release from the terminals of VTA neurons in the NAc (Xi and Stein, 2002a). However, this hypothesis does not explain how microinjection of a KOR agonist directly into the VTA produces aversion, nor does it address the function of dynorphinergic projections to the VTA. A necessary first step toward resolving these questions is to determine the direct actions of KOR agonists on the different classes of neurons in the VTA, including the subset that release DA. Here we report that KOR agonists directly inhibit a subset of DA-containing neurons in the VTA.

Experimental Procedures

Slice Preparation and Electrophysiology

20-36 day old male Sprague-Dawley rats were anesthetized with isoflurane, and the brains were removed. Horizontal brain slices (150-250 μm thick) containing the VTA were prepared using a vibratome (Leica Instruments, Germany). Slices were submerged in Ringer solution containing (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 35°C for at least 1 hour.

Individual slices were visualized under a Zeiss Axioskop with differential interference contrast optics and infrared illumination. Whole cell patch clamp recordings were made at 31°C using 2.5-4 MΩ pipettes containing (in mM): 123 K-gluconate, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 MgATP, and 0.3 Na3GTP (pH 7.2, osmolarity adjusted to 275). Biocytin (0.1%) was added to the internal solution for experiments in which cells were filled for later TH staining.

Recordings were made using an Axopatch 1-D (Axon Instruments, Union City, CA), filtered at 2 kHz and collected at 5 kHz using IGOR Pro (Wavemetrics, Lake Oswego, OR). Liquid junction potentials were not corrected during current- or voltage-clamp recordings. I_h currents were recorded by voltage clamping cells and stepping from -60 to -40, -50, -70, -80, -90, -100, -110, and -120 mV. Cells were recorded in current clamp mode (I=0) for experiments measuring spontaneous firing rates. In some experiments, 500 nM TTX was added to the bath solution to block neural activity after a stable 10 minute baseline was observed, and U69593 and DAMGO were subsequently added to this TTX solution. Current-voltage data was collected in voltage clamp by stepping from a holding potential of -60 mV to -40 mV and ramping down to -140 mV over a 2 second interval. Dose response data was collected with repeated applications of increasing doses of U69593 in each cell, and is reported as the percent of the inhibition produced by a maximal dose of 5 μM in each responding cell.

For data analysis, instantaneous firing rate was computed as the inverse of the inter-spike interval after each action potential. Results are presented as means ± SEM where appropriate. For each cell, the statistical significance of drug effects was tested with the paired student t-test, comparing the last 4 minutes of baseline to the last 4 minutes of drug application.

All drugs were applied by bath perfusion. Stock solutions were made and diluted in Ringer immediately prior to application. U69593 stock was diluted in 50% EtOH to a concentration of 1 mM; nor-Binaltorphimine (nor-BNI; 10 mM), and [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-Enkephalin (DAMGO; 1 mM), were diluted in H_2O; tetrodotoxin (TTX;

5mM) was diluted in DMSO. Agonists, antagonists, ATP, and GTP were obtained from Sigma Chemical (St. Louis, MO) or Tocris (Ballwin, MO).

Immunohistochemistry

Immediately after recording, slices were fixed for 2 hours in 4% formaldehyde. Slices were pre-blocked for 2 hours in PBS with 0.2% Triton X-100, 0.2% BSA and 5% normal goat serum. Rabbit anti-tyrosine hydroxylase antibody (1:100) was then added and the slices agitated at 4° C for 48 hours. Finally, slices were agitated with FITC anti-rabbit secondary antibody (1:100) and Texas Red avidin (11.0 microL /mL) at 4° C overnight. Cells were visualized with a Zeiss LSM 510 META microscope.

Biocytin, BSA, and normal goat serum were obtained from Sigma Chemical (St. Louis, MO). Texas red avidin was obtained from Jackson Immunoresearch (West Grove, PA), and rabbit anti-tyrosine hydroxylase antibody and FITC anti-rabbit secondary antibody were obtained from Chemicon (Temecula, CA).

Results

In these experiments, we studied postsynaptic KOR-mediated actions *in vitro* using current clamp recordings of neurons in horizontal rat brain slices of the VTA. We classified neurons according to their electrophysiological and pharmacological properties. Principal cells exhibit an I_h and long action potentials (Fig. 2-1A). Secondary cells lack an I_h, have shorter action potentials (Fig. 2-1B), and are generally considered to be GABAergic interneurons in both the VTA (Johnson and North, 1992a) and the neighboring substantia nigra zona compacta (SNC) (Grace and Onn, 1989; Lacey et al., 1989). Secondary cells are also inhibited by MOR agonists (Fig. 2-1B, Lacey et al.,

1989; Johnson and North, 1992a, b). While there is a consensus that the majority of VTA principal neurons are dopaminergic, Cameron et al. (1997) and Jones and Kauer (1999) have shown that a significant proportion of I_h-expressing VTA neurons are not immunoreactive for the enzyme tyrosine hydroxylase (TH). Furthermore, Cameron et al. (1997) identified a third class of neurons in the VTA which they call "tertiary" cells. Tertiary cells have soma morphology and action potential shape (Fig. 2-1C) very similar to those of principal cells, but tertiary neurons are inhibited by MOR agonists and serotonin and less than 1/3 of tertiary cells are TH(+) (Cameron et al., 1997). We classified I_h-expressing neurons as principal neurons if MOR agonists either had no effect or disinhibited the cells (Fig. 2-1A), and as tertiary if MOR agonists were inhibitory (Fig. 2-1C). We found no differences between tertiary and principal neurons for resting membrane potential, mean firing rate, or size of the I_h current (Table 2-1).

Principal neurons had an initial membrane potential of -44.4 +/- 0.8 mV and most (26/47) exhibited spontaneous activity. The KOR agonist U69593 inhibited 16 of 26 spontaneously active principal neurons (1 μM, Fig. 2-2A). About one third (8/21) of the principal neurons that were not firing spontaneously were hyperpolarized by U69593 (3.0 +/- 0.8 mV). Consistent with Johnson and North's (1992b) finding that MOR agonists disinhibit principal cells in the VTA *in vitro*, we observed an increase in spontaneous firing rate and/or depolarization in a subset (22/47) of principal neurons following application of DAMGO (3 μM, Table 2-1). This disinhibition has been attributed to a MOR agonist-induced inhibition of spontaneously active GABAergic interneurons, which allows principal neurons to fire more (Johnson and North, 1992b). Thus, our data suggest that at least some critical intra-VTA circuitry is preserved in these slices.

We found no evidence for desensitization of the action of U69593 on the timescale of these experiments. A KOR-mediated inhibition of similar magnitude was repeatedly evoked in a single cell after ample washout time had elapsed (2 applications of 5 minutes each per cell, n=3). Furthermore, cells maintained stable inhibitions during extended applications of U69593 (t>20 minutes, n=6). In KOR agonist-sensitive cells, the KOR selective antagonist nor-BNI (100 nM) completely blocked the effect of a subsequent application of U69593 (5 μM, n=4), confirming that the observed inhibition depends on the activation of KORs (Fig. 2-2B,C). The U69593-mediated inhibition was dose-dependent, and dose response experiments in KOR agonist-responsive neurons yielded an EC-50 of 42 nM (Fig. 2-2D).

Secondary neurons, which lack an I_h current and hyperpolarize in response to MOR agonists, could also be distinguished by their smaller size and relatively brief action potential. Secondary neurons were unaffected by U69593 (spontaneously active cells: mean change 0.2 +/- 0.3 Hz, n=6, quiescent cells: mean change 0.8 +/- 2.1 mV, n=3; Fig 2-3B, Table 2-1), confirming previous findings (Lacey et al., 1989; Johnson and North, 1992a). On the other hand, a significant proportion of tertiary cells, which have an I_h and hyperpolarize in response to MOR agonists, were responsive to KOR activation. Nine of 16 spontaneously firing tertiary neurons were inhibited by U69593 (Fig. 2-3C), and 7 of 9 non-firing tertiary neurons were hyperpolarized by U69593 (2.9 +/- 0.9 mV; Table 2-1).

The observation that DAMGO induces disinhibition in principal neurons indicates that some local circuitry is intact in the slice. Thus, in order to confirm that the inhibitory action we observed was due to a direct effect of the KOR agonist on the postsynaptic membrane of the recorded cell, it was necessary to measure the U69593 inhibition while

blocking neural activity. In the presence of TTX (500 nM), 4 of 10 neurons with an I_h (5 primary, 4 tertiary, 1 not classified) hyperpolarized when U69593 was added to the bath solution (5.0 +/- 0.9 mV, Fig. 2-4A). Additionally, the TTX itself had a depolarizing effect on the baseline membrane potential (5.3 +/- 0.4 mV after 5 minutes of bath application, P=0.01). This TTX-elicited depolarization implies that in this VTA slice preparation there is spontaneous activity of neurons that releases a hyperpolarizing neurotransmitter, such as GABA or dopamine, which acts on primary and tertiary neurons.

To test whether KOR activation inhibits these neurons by enhancing GIRK function, we applied a voltage ramp (step from -60mV to -40mV, ramp down to -140mV) before and during exposure to U69593. The current-voltage plot for the U69593-sensitive component of this voltage ramp showed a reversal potential of –93 mV, close to the calculated K^+ reversal potential (n=7, Fig. 2-4B) and had a diminished slope for positive current, suggesting the KOR is associated with a G protein-coupled inwardly rectifying potassium channel (GIRK).

Because some principal and most tertiary neurons are non-dopaminergic (Cameron et al., 1997), and a significant proportion of both cell types were not affected by KOR agonists, immunohistochemical identification of dopaminergic neurons is an essential step for interpreting the KOR agonist effect on VTA neurons. To determine whether the principal and tertiary cells inhibited by U69593 are indeed dopaminergic, biocytin was added to the internal pipette solution, and following recording the brain slices were fixed and stained for TH. Seventeen biocytin-filled VTA neurons were electrophysiologically defined, processed for TH reactivity, and recovered. Nine were principal neurons, seven

were tertiary, and one was secondary (Table 2-2). All 6 recovered cells that were inhibited by U69593 were TH(+) and had an I_h. Three of these were principal neurons (Fig. 2-5A). Six other principal neurons were unaffected by U69593, and one of these was TH(-) (Fig. 2-5B). Three of 7 recovered tertiary neurons were both KOR agonist-responsive and TH(+). The 4 KOR agonist-insensitive tertiary neurons were TH(-). Thus, KOR agonist-responsive neurons are a subset of dopaminergic neurons in the VTA, and most, if not all, KOR agonist-responsive neurons are TH(+) ($P<0.05$, fisher exact test), regardless of whether they are principal or tertiary cells. Importantly, our results demonstrate that there is a subset of VTA dopaminergic neurons (tertiary cells) that are inhibited by both MOR and KOR agonists.

Discussion

Our work shows that KOR selective agonists directly inhibit dopaminergic neurons in the VTA. Twenty-five percent of the principal neurons reported in this study exhibited both a KOR agonist-induced inhibition and a MOR agonist-induced disinhibition. This observation confirms and extends the work of Johnson and North (1992b), who originally described MOR agonist-mediated inhibition of GABAergic inputs to VTA principal cells. While the percentage of neurons exhibiting MOR agonist disinhibition is relatively small, it likely represents a significant underestimate of the proportion of cells that actually exhibit these opposing actions *in vivo*. It is probable that some intra-VTA circuitry is lost during the slicing procedure, and this loss decreases the number of cells showing a disinhibition with DAMGO. Additionally, in the Johnson and North study (1992b), the bath application of K^+ was required to increase GABA release sufficiently to demonstrate

disinhibition, whereas we observed the disinhibition without a manipulation to increase GABA release.

The opposing MOR and KOR agonist actions on principal cells suggest a mechanism for the opposing behavioral effects on place preference when these opioids are microinjected in the VTA (Bals-Kubik et al., 1993). Our results are also consistent with *in vivo* studies suggesting that changes in dopaminergic neuron firing encode the value of cue-associated outcomes during goal-directed behavior such that increases are positively reinforcing, and decreases lead to extinction of the behavior (Schultz, 1998). Thus, VTA injections of positively-reinforcing MOR agonists indirectly excite principal neurons through disinhibition, while aversive KOR agonists, at the same site, directly inhibit principal neurons.

Our immunohistochemical results specifically and selectively localize the postsynaptic KOR-mediated inhibition to dopaminergic neurons. Although direct KOR agonist inhibition did not occur in every VTA dopaminergic neuron, it was only observed in such neurons. This is of particular significance because previously suggested cellular markers, such as action potential shape and duration, firing rate, presence of I_h, and D2 agonist inhibition, do not in fact distinguish dopaminergic from non-dopaminergic VTA neurons (Grace and Onn, 1989; Johnson and North, 1992a; Cameron et al., 1997; Jones and Kauer, 1999). Our results reinforce the point that there are no established electrophysiological or pharmacological properties that specifically delineate dopaminergic neurons from non-dopaminergic neurons in the VTA. Clearly, no current classifications of VTA neurons do justice to the heterogeneity of cell responses that are observed. For example, in the current study, we found I_h neurons that fell into all four

possible categories: MOR agonist-inhibited, KOR agonist-inhibited, inhibited by both MOR and KOR agonists, and inhibited by neither. Additionally, the approach taken by Cameron et al. (1997) and used in this study, i.e. delineating a subgroup of neurons (tertiary cells) expressing an I_h and being directly inhibited by MOR agonists, is a demonstrably inadequate indicator for non-dopaminergic VTA neurons. This issue is highlighted by the fact that half of the TH(+) neurons that were inhibited by a KOR agonist were also inhibited by a MOR agonist. Clearly, a more reliable and meaningful characterization of cell types in the VTA is needed. One intriguing possibility is that consistent pharmacological differences will coincide with different projection targets and/or sources of afferent input. Such information will be critical for gaining a more robust understanding of the contribution of dopaminergic VTA neurons to motivation and reward.

Previous studies have demonstrated a decrease in dopamine release in VTA cell cultures when KOR agonists are applied (Ronken et al., 1993; Dalman and O'Malley, 1999). However, these cell culture experiments leave open to question the cell types and proportion of cells affected. Furthermore, KOR agonists bath applied to cultured neurons could act at either the cell body or the dopaminergic terminals, or both. In our whole cell slice recordings, we show that KOR agonists directly activate receptors on the soma-dendritic region of a subset of dopaminergic neurons, which in turn inhibit these neurons through the activation of a GIRK channel.

Although we found a clear inhibition of dopaminergic neurons by KOR agonists, Spanagel et al. (1992) and Devine et al. (1993a) did not observe a change in the DA concentration detected by microdialysis in the NAc following microinjection of KOR

agonists into the VTA.. One possible confound in these dialysis experiments is that the measurements were made in anaesthetized animals, and it is likely that the firing patterns of the VTA neurons were different from those in awake, behaving rats given KOR agonists in the conditioned place preference paradigm. If VTA dopaminergic neurons are not spontaneously active in anaesthetized rats, it would not be possible to demonstrate inhibition of firing by soma-dendritic application of KOR agonists. On the other hand, microinjections of KOR agonists in the VTA have been reported to decrease extracellular dopamine in the NAc after it has been increased by systemic administration of haloperidol (Leyton et al., 1992). Furthermore, KOR agonists delivered by microdialysis into the substantia nigra of awake rats significantly decrease dopamine release in the neostriatum (You et al., 1999). Behaviorally, 6-OHDA lesions of the NAc block place aversion elicited by systemic KOR agonists (Shippenberg et al., 1993). These studies indicate that modulating the dopaminergic signal to the NAc with KOR agonists in the VTA is not only possible, but may be necessary for the expression of behavioral effects of KOR agonists in the VTA.

In conclusion, we demonstrate that KOR agonists directly inhibit a subset of dopaminergic VTA neurons. At least some of these neurons are also disinhibited by MOR agonists. The direct KOR-mediated inhibition of principal neurons which are also disinhibited by MOR agonists is a motif that has been found elsewhere in the central nervous system (Pan, 1998), although other arrangements have been reported (Ackley et al., 2001; Marinelli et al., 2002). Of the three VTA cell types studied here, it is only dopaminergic principal neurons that are both inhibited by KOR agonists and disinhibited by MOR agonists. Thus, principal cell opioid responses provide the simplest explanation

for the opposing behavioral effects elicited by MOR and KOR agonists injected into the VTA. Secondary cells are unaffected by KOR agonists, and tertiary cells are inhibited by both MOR and KOR agonists, and thus neither is a likely candidate for providing preference-related information. The opposing behavioral effects of MOR and KOR agonists acting in the VTA can be explained by the segregation of MORs and KORs onto distinct but interconnected subpopulations of neurons that produce outputs of opposite sign. Our data extend a growing body of evidence that endogenous KOR agonists play an important general role in modulating circuits implicated in motivation and reinforcement (Shippenberg et al., 2001). By directly inhibiting dopaminergic VTA neurons, KOR agonists can powerfully control the motivational effects of both natural and drug rewards.

Table 2-1: Principal and Tertiary VTA neurons have similar electrophysiological properties but respond differently to μ and κ opioids.

Cell Type	Mean firing rate (Hz)	I_h size (pA)	Inhibited by U69593 only*	Inhibited by DAMGO only*	Inhibited by U69593 & DAMGO*	Disinhibited by DAMGO only*	Inhibited by U69593 & disinhibited by DAMGO*
Principal (n=47)	1.3 ± 0.2	-240 ± 30	12	X	X	10	12
Secondary (n=9)	3 ± 1	1 ± 2	X	9	0	X	X
Tertiary (n=25)	1.2 ± 0.2	-280 ± 40	X	9	16	X	X

Summary of current clamp recordings in both spontaneously firing and quiescent VTA neurons. Each drug was bath-applied independently for 5 minutes. Note: By definition, all secondary and tertiary cells were inhibited by DAMGO. Neuronal properties that are not compatible with our cell type definitions are denoted by "X." (See Results). *Effect criterion is paired t-test of baseline to the last 4 minutes of drug application within each cell, $P<0.05$.

Table 2-2: Dopamine-containing neurons are inhibited by κ and μ opioids.

	κ inhibition only*	κ and μ inhibition*	μ inhibition only*	Neither κ nor μ*
TH (+) (n=11)	3	3	0	5
TH (-) (n=6)	0	0	5**	1

Summary of current clamp recordings in both spontaneously firing and quiescent VTA neurons loaded with biocytin during recordings and stained for TH afterwards. Each drug was bath-applied independently for 5 minutes. *Effect criterion is paired t-test of baseline to the last 4 minutes of drug application within each cell, $P<0.05$. **Data include four tertiary cells and one secondary cell.

Figure Legends

Figure 2-1. VTA neurons can be classified using electrophysiological and pharmacological means. A, A typical principal neuron exhibits an I_h current sag in response to hyperpolarizing voltage steps (left), has a long action potential (center), and is disinhibited by the MOR agonist DAMGO (3 μM) (right). B, A typical secondary neuron lacks an I_h, has a short action potential, and is hyperpolarized by DAMGO (3 μM). C, A typical tertiary cell exhibits an I_h, has a long action potential, and is inhibited by DAMGO (3 μM).

Figure 2-2. Kappa opioids in the VTA are inhibitory through activation of KOR. A, The firing rate of spontaneously active principal neurons (n=26) is inhibited by bath application of U69593 (1 μM) but not DAMGO (3 μM). B, In a single cell, the U69593-evoked (5 μM) inhibition is completely blocked during re-application of U69593 (5 μM) in the presence of the KOR selective antagonist nor-BNI (100 nM). C, nor-BNI (100 nM) blocked the U69593 (5 μM) inhibition in all tested cells (n=4, error bars indicate SEM). D, Dose-response curve for U69593 inhibition (n=4 for each point, error bars indicate SEM).

Figure 2-3. Spontaneous activity of principal and tertiary neurons is inhibited by U69593. Distributions of inhibition by U69593 (1 μM) in all spontaneously active principal (A), secondary (B), and tertiary (C) neurons. A significant change in the firing rate due to drug compared to baseline within each cell is indicated by a darkened bar, $P<0.05$.

Figure 2-4. KOR agonist inhibition of VTA neurons is postsynaptic and mediated by GIRK activation. A, In the presence of TTX (500 nM) to block synaptic transmission, U69593 causes hyperpolarization in an example cell. B, In recordings made without TTX, the difference between control and U69593 (1 μM) current-voltage curves during a voltage ramp test shows inward rectification and a reversal potential of -93 mV (n=7, 95% confidence interval indicated in grey).

Figure 2-5. KOR activation inhibits dopaminergic neurons. A, A principal neuron filled with biocytin (red) during electrophysiological recording is co-stained with TH antibody (green) (left). This neuron exhibited an I_h current when tested with 200 msec voltage steps from -60 mV to -50, -70, -90, -100, and -120 mV (center). The KOR agonist U69593 (1 μM) inhibited spontaneous activity of this neuron held in current clamp at I=0 (right). B, A principal neuron filled with biocytin (red) is not co-stained with TH antibody (green). This neuron also exhibited an I_h current. U69593 (1 μM) had no effect on the firing rate of this neuron.

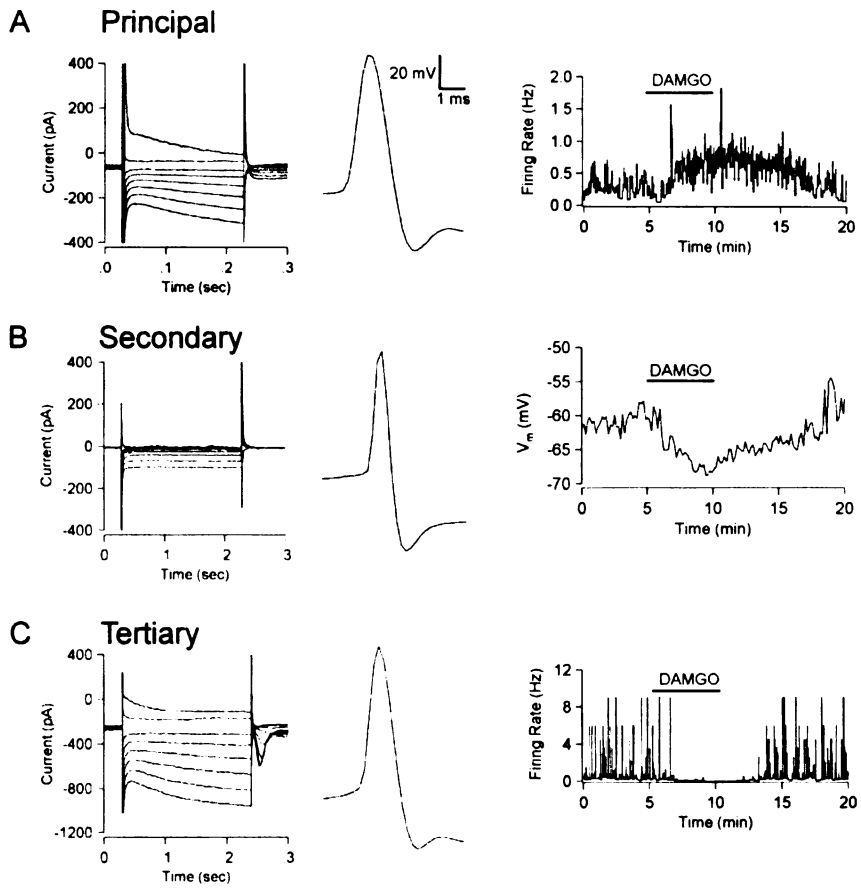
A
Principal
400
200
0
-200
-400
Current (pA)
0
1
2
3
Time (sec)
20 mV
1 ms
2.0
1.5
1.0
0.5
0.0
Firing Rate (Hz)
DAMGO
0
5
10
15
20
Time (min)
B
Secondary
400
200
0
-200
-400
Current (pA)
0
1
2
3
Time (sec)
-50
-55
-60
-65
-70
V_m (mV)
DAMGO
0
5
10
15
20
Time (min)
C
Tertiary
400
0
-400
-800
-1200
Current (pA)
0
1
2
3
Time (sec)
12
8
4
0
Firing Rate (Hz)
DAMGO
0
5
10
15
20
Time (min)

Figure 2-1

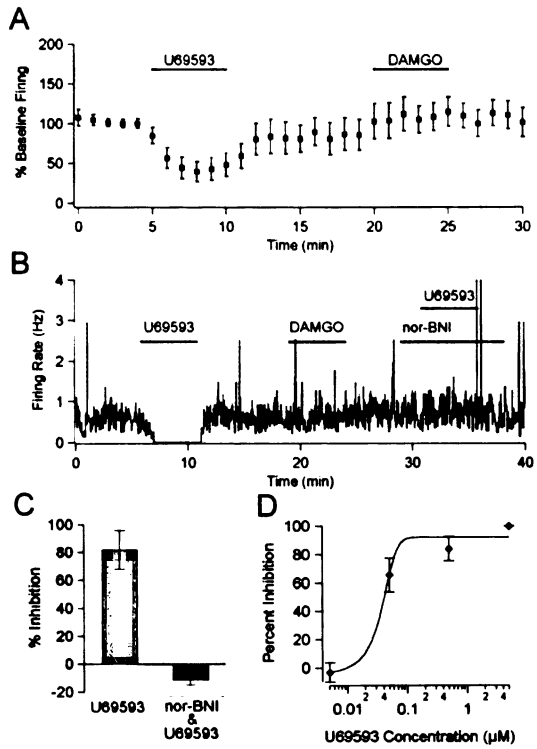

Figure 2-2

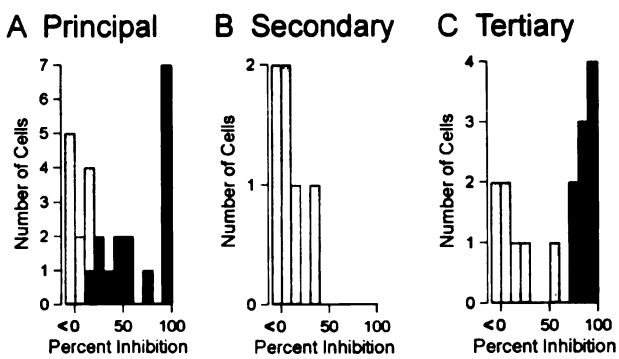

Figure 2-3

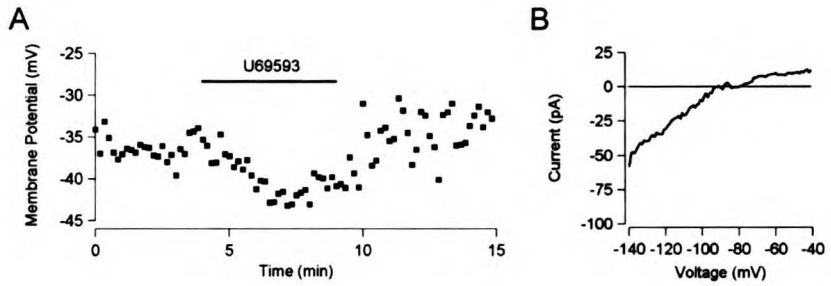

Figure 2-4

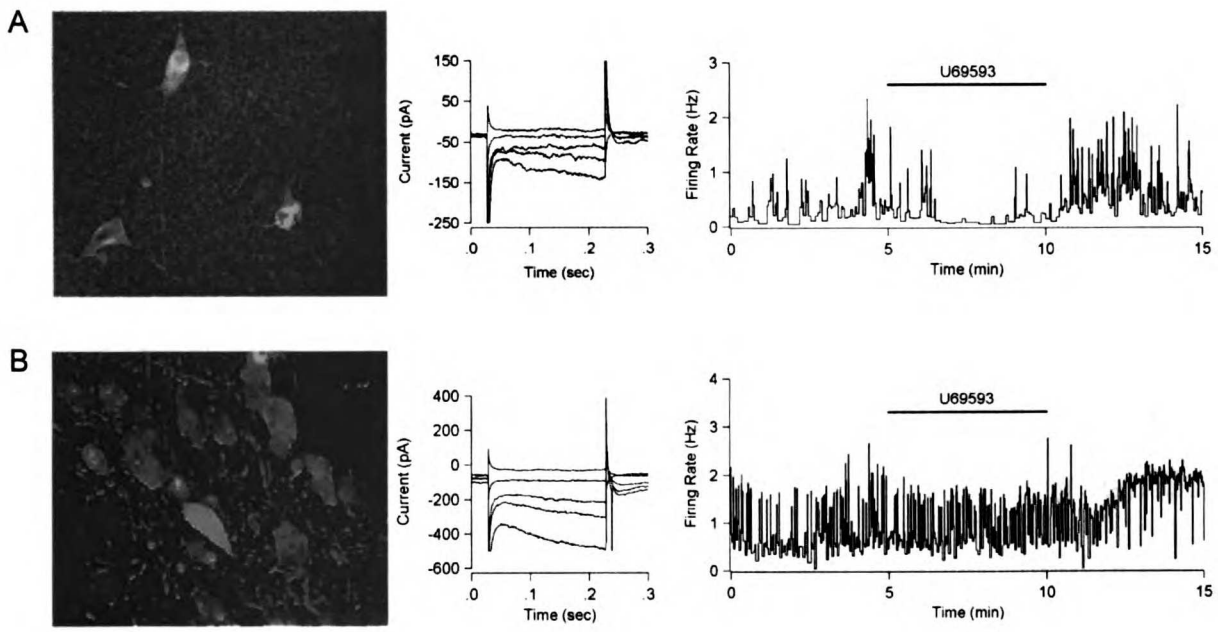

Figure 2-5

Chapter 3

Both Kappa and Mu Opioid Agonists Inhibit Glutamatergic Input to Ventral Tegmental Area Neurons

Abstract

Mu and Kappa opioid (MOP and KOP) agonists produce powerful and largely opposing motivational actions when microinjected into the midbrain ventral tegmental area (VTA). There are several classes of neuron in the VTA: principal neurons, (mostly dopamine-containing and disinhibited by MOP agonists), secondary neurons (presumed GABAergic interneurons) and tertiary neurons (of which about 40% are dopaminergic). Secondary and tertiary neurons are directly hyperpolarized by MOP agonists. KOP agonists hyperpolarize about half of principal and tertiary cells but have no direct effects on secondary cells. In contrast to the fairly complete analysis of postsynaptic opioid effects, presynaptic MOP and KOP control of excitatory glutamatergic transmission onto the different classes of VTA neurons has not been systematically studied. To address this issue we used whole cell recording in VTA slices and measured excitatory postsynaptic currents (EPSCs). We confirmed that MOP agonists inhibit glutamate release in principal (48.5%) and secondary (53.3%) cells and demonstrated that the KOP agonist U69593 produces a small reduction in EPSC amplitude in principal neurons (13.4 %). U69593 also robustly inhibited EPSCs in secondary cells (46.1 %), and, in this cell class, the magnitude of this inhibition was positively correlated with that produced by the MOP

agonist. Finally, EPSCs in tertiary neurons were significantly inhibited by both U69593 (35.1 %) and DAMGO (33.5 %). In addition to their powerful direct inhibition of VTA neurons, this presynaptic modulation of excitatory inputs to VTA neurons permits opioidergic control to be exerted selectively upon different inputs to VTA.

Introduction

The ventral tegmental area (VTA) has been implicated in the rewarding and aversive motivational actions of a variety of drugs including opioid agonists. Both κ opioid (KOP) (1993; Arvidsson et al., 1995; Mansour et al., 1996) and μ opioid (MOP) (Garzon and Pickel, 2001; Svingos et al., 2001) receptors are present in significant density in the VTA. Further, microinjections of both MOP and KOP receptor agonists directly into the VTA produce robust behavioral responses: the MOP agonist DAMGO injected directly into the VTA produces conditioned place preference (CPP) (Phillips and LePiane, 1980; Bals-Kubik et al., 1993; Nader and van der Kooy, 1997); the KOP agonist U50488 produces conditioned place aversion (CPA) (Bals-Kubik et al., 1993).

Glutamate transmission within the VTA is required for the motivational properties of opioids (e.g. Cornish et al., 2001; Xi and Stein, 2002b; Harris and Aston-Jones, 2003). In the VTA, glutamatergic inputs are derived from neurons in the medial prefrontal cortex (mPFC), pedunculopontine region, and the subthalamic nucleus (Paxinos, 1995). There is also indirect evidence that lateral hypothalamic projections to the VTA contain glutamate (Chou et al., 2001; Rosin et al., 2003). In normal VTA function, glutamate can induce phasic firing of dopaminergic neurons (Floresco et al., 2003) through the activation of NMDA receptors (Johnson et al., 1992; Overton and Clark, 1997), and this

effect is facilitated by group 1 metabotropic glutamate receptor activation (Zheng and Johnson, 2002). VTA glutamate also increases extracellular DA in the NAc through a non-NMDA mechanism (Mathe et al., 1998), suggesting a role for AMPA receptor activation in the VTA.

Essential to determining how MOP and KOP receptor agonists in the VTA produce their behavioral actions is elucidating their synaptic effects at the cellular level. MOP receptor agonists postsynaptically inhibit putative gamma-aminobutyric acid (GABA) interneurons, an action proposed to disinhibit principal (mostly dopaminergic) cells (Johnson and North, 1992b; Margolis et al., 2003). Tertiary neurons (about 40% dopaminergic) are also postsynaptically inhibited by MOP receptor agonists (Cameron et al., 1997). We recently reported that KOP receptor agonists postsynaptically inhibit a subset of dopaminergic neurons, some of which were also inhibited by MOP receptor agonists (Margolis et al., 2003).

Despite the evidence that VTA glutamatergic transmission is critical for reward and motivation, our understanding of presynaptic control of glutamate release by opioids is incomplete. For instance, MOP receptor agonists presynaptically inhibit the release of glutamate in synapses onto principal and secondary cells (Bonci and Malenka, 1999; Manzoni and Williams, 1999), but their effect in tertiary neurons is not known. To extend our understanding of opioid control in the VTA, we examined the presynaptic effects of both KOP and MOP receptor agonists on synaptic glutamate release onto each VTA cell type in this study. Furthermore, we directly compared KOP and MOP effects within and across individual neurons and neuron types.

Methods

20-36 day old male Sprague-Dawley rats were anesthetized with isofluorane, and the brain was removed. Horizontal brain slices (200 microm thick) containing the VTA were prepared using a vibratome (Leica Instruments, Germany). Slices were submerged in Ringer solution containing (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO4, 1.0 NaH2PO4, 2.5 CaCl2, 26.2 NaHCO3, and 11 glucose saturated with 95% O2-5% CO2 and allowed to recover at 32°C for at least 1 hour.

Individual slices were visualized under a Zeiss Axioskop with differential interference contrast optics and infrared illumination. Whole cell patch clamp recordings were made at 31°C using 2.5-4 MΩ pipettes containing (in mM) 123 K-gluconate, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 MgATP, and 0.3 Na3GTP (pH 7.2, osmolarity adjusted to 275).

Recordings were made using an Axopatch 1-D, filtered at 2 kHz and collected at 5 kHz using IGOR Pro (Wavemetrics, Lake Oswego, OR). I_h currents were recorded by voltage clamping cells and stepping from -60 to -40, -50, -70, -80, -90, -100, -110, and -120 mV. Series resistance and input resistance were sampled throughout voltage clamp experiments with 4 mV, 200 msec depolarizing steps once every 10 seconds. For cell typing purposes, every neuron was tested for postsynaptic DAMGO (3 microM) or 5-CT (500 nM) responsiveness. Cells were recorded in voltage clamp mode (V=-70mV) while measuring EPSCs. All EPSCs were measured in the presence of picrotoxin (100 mM). Stimulating electrodes were placed 60-150 microns rostral to the patch clamped cell. Paired pulses 50 msec apart and were delivered once every 10 seconds. The EPSC amplitude was calculated by comparing a 2 msec period around the peak to a 2 msec

interval just prior to stimulation. The paired pulse ratio was calculated by dividing the amplitude of the second EPSC by that of the first trial by trial, and then averaged across trials. Spontaneous events were identified by searching the smoothed first derivative of the data trace for values that exceeded a set threshold, and these events were then visually confirmed.

Results are presented as means +/- SEM where appropriate. For each condition, significance of effects were tested with the paired student t-test, comparing the average value for the 4 minutes of baseline just preceding drug application to the average value during the last 4 minutes of drug application.

All drugs were applied by bath perfusion. Stock solutions were made and diluted in Ringer immediately prior to application. U69593 stock was diluted in 50% EtOH to a concentration of 1 mM; nor-Binaltorphimine (nor-BNI; 10 mM), 5-CT (1mM), and [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-Enkephalin (DAMGO; 1 mM), were diluted in H_2O. Picrotoxin stock was diluted in DMSO (100 mM). Agonists, antagonists, ATP, and GTP were obtained from Sigma Chemical (St. Louis, MO) or Tocris (Ballwin, MO).

Results

Whole-cell voltage-clamp recordings were made from neurons in the VTA. Pharmacologically-isolated excitatory postsynaptic currents (EPSCs) were electrically evoked and we confirmed that this evoked EPSC current was due to AMPA activation by blocking the response with the non-NMDA antagonist DNQX (10 μM, n=3, Fig. 3-1A).

We were interested in whether opioids differentially alter glutamatergic transmission onto different cell types in the VTA. Therefore, we classified neurons according to their electrophysiological and pharmacological properties. Principal cells exhibit an I_h and

long action potentials (Fig. 3-1B). Most of these neurons are dopaminergic. Secondary cells lack an I_h, have shorter action potentials (Fig. 3-1C), and are generally considered to be GABAergic interneurons (Johnson and North, 1992a). Secondary cells are also directly inhibited by MOP receptor agonists through GIRK activation (Fig. 3-1C, Lacey et al., 1989; Johnson and North, 1992a, b). Tertiary cells have soma morphology and action potential shape (Fig. 3-1D) very similar to those of principal cells, however in contrast to principal cells, tertiary neurons are directly inhibited by both MOP receptor agonists and serotonin (Cameron et al., 1997). Whereas about 80% of principal neurons are TH(+), only about 40% tertiary cells are TH(+). To distinguish primary neurons from tertiary neurons, at the end of each experiment, we applied DAMGO while recording the membrane potential in current clamp.

The effects of both KOPs and MOPs on EPSC amplitude were examined in each cell type in the VTA. Because pilot experiments showed that inhibition by the KOP receptor agonist U69593 persisted for at least 15 minutes after washout commenced (1 μM, n=6, Fig. 3-2A), the KOP receptor selective antagonist nor-BNI (100 nM) was used to reverse the KOP mediated inhibition in later experiments. Application of nor-BNI prior to U69593 completely blocked the KOP agonist effect (n=3, Fig. 3-2B). Because DAMGO was applied a second time to identify the cell type in current clamp, no antagonist was used in most experiments to reverse the prolonged presynaptic MOP receptor activation response. However, the application of the MOP receptor selective antagonist CTAP (500 nM) reverses the DAMGO (3 μM) effect (n=4, Fig. 3-2C).

The EPSC response to U69593 (1 μM) and DAMGO (3 μM) was tested in each cell (Fig. 3-3A). U69593 produced a modest reduction in EPSC amplitude in principal

neurons (13.4%), smaller than the DAMGO effect in the same neurons (41.4%, n=11). In contrast, in secondary cells, EPSCs were robustly inhibited by both U69593 (46.1 %) and DAMGO (53.3 %, n=10). EPSCs in tertiary neurons were also significantly inhibited by both U69593 (35.1 %) and DAMGO (33.4 %, n=9) (Fig. 3-3B,C).

Comparing cell types, the observed U69593 effect was significantly larger in secondary and tertiary neurons than in principal neurons (Fig. 3-3B). While there is a trend towards a difference between secondary and tertiary cell EPSC inhibition, KOP responses are not significantly different in these populations. The DAMGO EPSC inhibition was not significantly different among cell types (Fig. 3-3C). For both opioid types, the largest inhibitions were observed in secondary neurons, and only secondary cells have a significant correlation between KOP and MOP EPSC inhibitions (Fig. 3-3D). It is interesting to note that the EPSCs in a subset of secondary neurons (3/10) were inhibited more than 75% by both U69593 and DAMGO, a much larger effect than that observed in any principal or tertiary neuron. In these experiments there were many stimulations that did not evoke any EPSC at all when opioids were added to the superfusion solution. Therefore, while the mean EPSC inhibition by MOP receptor agonists in secondary cells may not be different from that in principal and tertiary cells, and KOP effects on secondary and tertiary EPSCs have similar averages, there does seem to be an underlying difference in the maximum magnitude and distribution of EPSC inhibition by KOP and MOP opioids in secondary neurons.

To confirm that these effects were presynaptic, we examined changes in the paired pulse ratio (PPR) due to each drug. The PPR is determined by stimulating the afferents twice over a short time interval (50 msec), and dividing the amplitude of the second

evoked EPSC by that of the first. We found no significant differences between the baseline PPRs of the different cell types (Fig. 3-4A). A drug-induced decrease in the probability of release is correlated with an increase in the paired pulse ratio (Manabe et al., 1993). Figure 3-4B shows example traces from each cell type comparing baseline to U69593 and DAMGO evoked EPSCs. While the drug-induced changes in PPR varied greatly across cells and cell types, there was a significant overall linear correlation between the magnitude of EPSC inhibition and the change in PPR for both U69593 (Fig. 3-4C, principal n=11, secondary n=10, tertiary n=9) and DAMGO (Fig. 3-4D, principal n=11, secondary n=10, tertiary n=8), suggesting a presynaptic site of action for the observed changes in EPSC amplitude.

For a second method of confirmation of a presynaptic site of action for the observed effects, we monitored spontaneous excitatory events (sEPSCs). In example traces from a secondary cell, sEPSCs are present in a 1 second baseline trace, and fewer occur in the presence of U69593 (1 μM, Figure 3-5A). Cumulative plots for this same neuron show that the amplitudes of the sEPSCs do not change in the presence of the KOP receptor agonist (Fig. 3-5B), but there is a shift to the right of the inter-event interval caused by the inhibition of spontaneous events (Fig. 3-5C).

This pattern was observed across cell types in response to both U69593 and DAMGO. No difference was found between the baseline frequency of sEPSCs and miniature events recorded in the presence of TTX (500 nM) to block spontaneous neural activity (n=4, P>0.6, Fig. 3-5D). There was some variability between cells in sEPSC frequency, but no differences in baseline frequencies between cell types (principal: 1.4 +/- 0.5 Hz, n=8; secondary: 3.8 +/- 1.3 Hz, n=8; tertiary: 2.4 +/- 0.5 Hz, n=7; Fig. 3-5D).

The frequency of sEPSCs significantly decreased from baseline across all cell types in response to both U69593 ($P<0.001$, Fig. 3-5E) and DAMGO ($P<0.05$, Fig. 3-5F). The amplitudes of sEPSCs, however, showed neither a baseline difference across cell types, nor changes in response to drug applications (Fig. 3-5G,H). The presence of a decrease in sEPSC frequency and a lack of an effect on sEPSC amplitude suggest a decrease in release probability in response to both drugs.

Discussion

Our results demonstrate that glutamate release in each class of VTA neuron is inhibited presynaptically by both KOP and MOP receptor agonists. However, these actions are not of equivalent magnitude. While KOP EPSC inhibition is larger in secondary and tertiary cells than it is in principal neurons, there was no difference in the magnitude of MOP EPSC inhibition across cell types. Additionally, only in secondary cells is there a significant correlation between the magnitudes of the KOP- and MOP-induced EPSC inhibitions. The KOP-mediated inhibition was confirmed as a presynaptic effect on glutamate release by observing both that an increase in the PPR correlated with EPSC inhibition, and sEPSC frequency decreased with the application of U69593, but no drug induced change in amplitude of spontaneous events were observed.

Previous studies have reported findings somewhat discordant with what is reported here. While our results confirm previous reports of presynaptic modulation of glutamate release by MOP receptor agonists in principal and secondary neurons (Bonci and Malenka, 1999; Manzoni and Williams, 1999), the presynaptic KOP effect reported here was not observed in a previous investigation of glutamate release onto principal neurons in the VTA (Manzoni and Williams, 1999). Although the KOP effect in principal

neurons reported here is smaller than that in the other cell types, the effect is not only confirmed by PPR and sEPSC measurements, but is also reversed by nor-BNI, confirming specific activation of the KOP receptor. Bonci and Malenka (1999) found a difference between the baseline PPR of I_h and non-I_h neurons which was not observed here. It is possible that even though the inter-stimulus interval used to test PPR in these experiments was the same as that used in the Bonci and Malenka study, some other technical difference between the experiments, such as stimulus strength, stimulus duration, or stimulating electrode distance from the cell body, may be responsible for the different baseline observations.

Unlike the combinations of postsynaptic KOP and MOP receptors that are differentially expressed in the different VTA cell classes, the similarity across cell types of presynaptic inhibition of glutamate release by both KOP and MOP receptor agonists suggests a broader functional range of opioid control of VTA neuronal activity. Similar presynaptic KOP inhibition of glutamate release onto cell types having different postsynaptic opioid responses has also been observed in the nucleus raphe magnus (Bie and Pan, 2003). Presumably, glutamate activation of principal, secondary, and tertiary neurons would have very different effects on VTA output. Interestingly, both opioid types investigated here inhibit all of these excitatory signals. Given these findings an important unknown is the location and timing of release of KOP and MOP receptor endogenous ligands. Enkephalin projections from the ventral pallidum and endomorphin projections from the hypothalamus that activate MOP receptors are most likely activated by different events than those that lead to the release of the KOP receptor ligand dynorphin from terminals of neurons located in the nucleus accumbens, lateral

hypothalamus, and amygdala (Fallon et al., 1985; Paxinos, 1995; Chou et al., 2001; Greenwell et al., 2002). Therefore, the KOP and MOP signals that are so similar *in vitro* may be functionally very different in the whole animal.

Postsynaptic inhibitions by both KOP and MOP receptor agonists in the VTA have previously been examined in the cell types investigated here (Cameron et al., 1997; Margolis et al., 2003). Postsynaptically, only subsets of dopaminergic principal and tertiary neurons are inhibited by KOP receptor agonists, while, by definition, all secondary and tertiary cells are inhibited by MOP receptor agonists. Thus, concurrent KOP- or MOP-induced presynaptic inhibition of glutamate release and postsynaptic hyperpolarization would be synergistic in secondary and tertiary neurons. Since principal and tertiary neurons fire in the absence of synaptic input, opioids can modify VTA output even when there is no excitatory signal coming into the VTA. There may also be an anatomical differentiation of these signals. Endogenous opioids may have a limited radius of effect when released and therefore presynaptic and postsynaptic KOP and MOP receptors could be activated independently *in vivo*. Such mechanisms would account for the seeming contradiction that MOP receptor agonists both inhibit excitatory input and indirectly disinhibit principal neurons.

Another mechanistic difference between pre- and postsynaptic opioid effects in the VTA could arise from the marked contrast in the timing of the postsynaptic and presynaptic opioid effects in the VTA observed *in vitro*. Whereas the postsynaptic effects washout almost immediately upon removal of the agonist from the bath perfusion (Margolis et al., 2003), the presynaptic effects of both KOP and MOP are long-lasting in the slice if an antagonist is not applied. While it is possible that this difference is an

artifact of the kinetics of drugs washing out of different structures in the slice, it is also possible that the cascade of events that follow receptor activation at pre- and postsynaptic membranes have characteristically different timescales. For instance, if receptors on terminals are internalized when activated and those on cell bodies are not, the presynaptic effects of opioids in the VTA could be prolonged compared to the postsynaptic effects. The antagonist's rapid reversal of the agonist response makes receptor internalization an unlikely mechanism for the prolonged effect, however the relatively rapid response to both agonist and antagonist wash-in also suggests that retarded diffusion through the slice is not the cause of the prolonged presynaptic effect.

The source of the differences in the responses to opioids reported here and their possible functional implications are unclear. Since in most cases, especially in the principal neurons, inhibition of glutamatergic inputs by KOP ligands was only partial, it is likely that not all glutamatergic terminals bear the KOP receptor. Thus, it is tempting to hypothesize that EPSC modulation by opioids across cell types varies depending on the source of glutamatergic afferents. For instance, Carr and Sesack (2000) have shown that glutamate afferents from the mPFC to the VTA synapse selectively on DA neurons that project back to the mPFC and GABAergic neurons that project to the NAc. Therefore, some combination of the pedunculopontine region, subthalamic nucleus, or hypothalamus likely provides major glutamate input to the non-dopaminergic neurons that comprise 60% of the VTA projection to the mPFC, the dopaminergic neurons that comprise 80% of the VTA projection to the NAc, and possibly VTA neurons that project to other targets such as the amygdala and hippocampus (Swanson, 1982). However, since in other brain regions a single axon can give rise to multiple excitatory synapses

with significantly different properties (Maccaferri et al., 1998; Markram et al., 1998; Scanziani et al., 1998), we cannot conclude that the observed differences are due to the differential anatomical origins of the glutamatergic afferents. Further work needs to be done in order to discern if there is indeed an anatomical correlate to the effects observed here.

In conclusion, we demonstrate that KOP and MOP receptor agonists inhibit glutamatergic input onto all neuron types in the VTA. While the KOP-mediated presynaptic effect in principal neurons was smaller than the effect in secondary and tertiary neurons, the MOP effect in all cell types was the same magnitude. Presynaptic regulation of synaptic transmission by opioids in the VTA provides a mechanism for selective control of specific inputs to VTA neurons. How these presynaptic effects interact with the postsynaptic inhibitions by MOP and KOP receptor activation in the VTA not only depend on whether or not the glutamatergic afferents are active when opioid ligands are present, but may also depend on the source of the glutamate, or the projection target of the postsynaptic neuron. The modulation of glutamate release by KOP and MOP receptor agonists reported here informs our understanding of the VTA, and is an important key to understanding normal VTA function and the influence of endogenous opioids on VTA output.

Figure Legends

Figure 3-1. VTA neurons can be classified using electrophysiological and pharmacological means. A, VTA neurons exhibit a non-NMDA mediated evoked EPSC that is blocked by the antagonist DNQX (10 μM) B, A typical principal neuron exhibits an I_h current sag in response to hyperpolarizing voltage steps (left), has a long action potential (center), and is disinhibited by the MOP agonist DAMGO (3 μM) (right). C, A typical secondary neuron lacks an I_h, has a short action potential, and is hyperpolarized by DAMGO (3 μM). D, A typical tertiary cell exhibits an I_h, has a long action potential, and is inhibited by DAMGO (3 μM).

Figure 3-2. EPSCs in VTA neurons are inhibited by both kappa opioid (KOP) and MOP receptor agonists. A, In an example experiment, evoked EPSCs are inhibited by bath application of the KOP receptor agonist U69593 (1 μM). This effect persists for at least 10 minutes after washout of the drug commences. B, In another experiment, this effect is completely blocked in the presence of the KOP receptor selective antagonist nor-BNI (100 nM). C, The MOP receptor agonist DAMGO (3 μM) inhibits EPSC amplitude in a sample experiment. This effect persists until the MOP receptor selective antagonist CTAP (500 nM) is is added to the superfusion solution.

ANOVA *P<0.05; **P<0.01.

Figure 3-3. EPSC inhibition by KOP and MOP receptor agonists show differences across cell types. A, Evoked EPSCs in principal (n=11), secondary (n=10), and tertiary (n=9)

neurons are inhibited by the KOP receptor agonist U69593 (1 μM). This inhibition is reversed by the KOP receptor selective antagonist nor-BNI (100 nM). DAMGO (3 μM) inhibits EPSC amplitude in the same cells of each cell type. B, The average EPSC inhibition by the KOP receptor selective agonist U69593 (1 μM) in principal neurons is smaller than that observed in secondary and tertiary neurons. There is no difference in the average EPSC inhibition by the MOP receptor selective agonist DAMGO (3 μM) across cell types. D, The distribution of all cells in which both a U69593 and DAMGO response were tested yields a significant linear correlation between EPSC inhibition by KOP and MOP receptor agonists only in secondary neurons. The greatest inhibitions by both U69593 and DAMGO were also observed in secondary neurons.

Figure 3-4. Changes in paired pulse ratio in VTA neurons due to KOP and MOP receptor agonists are correlated with EPSC inhibition. A, There is no difference in the baseline paired pulse ratio of each cell type. B, Example traces from principal, secondary, and tertiary neurons of baseline paired EPSCs and those evoked in the presence of either U69593 or DAMGO, all exhibiting an increase in the paired pulse ratio in response to drug application. C, There is a significant linear correlation ($P<0.05$) between the magnitude of EPSC inhibition by U69593 and the change in the paired pulse ratio caused by the drug in principal (n=11), secondary (n=10) and tertiary (n=9) neurons. D, Similarly, there is a significant linear correlation ($P<0.05$) between DAMGO-induced EPSC inhibition and paired pulse ratio change in principal (n=11), secondary (n=10), and tertiary (n=8) neurons.

Figure 3-5. The frequency, but not amplitude, of sEPSCs is diminished by both KOP and MOP receptor agonists. A, Sample traces of spontaneous activity recordings in a secondary neuron during baseline and U69593 application (1 μM). B, In the same neuron, there is no difference between sEPSC amplitudes during baseline and in the presence of U69593. C, U69593 in this cell shifts the inter-event interval to the right of baseline. D, No difference was observed in the baseline sEPSC frequency across cell types. E, sEPSC frequency in principal (n=8), secondary (n=8) and tertiary (n=7) neurons was significantly decreased by the KOP receptor agonist U69593 (1 μM). F, sEPSC frequency was also significantly decreased across all cells during the application of the MOP receptor agonist DAMGO (3 μM). G, The baseline amplitude of sEPSCs did not differ across cell types. Amplitude was also not affected by U69593 (1 μM). H, Bath application of DAMGO (3 μM) also had no effect on sEPSC amplitude.

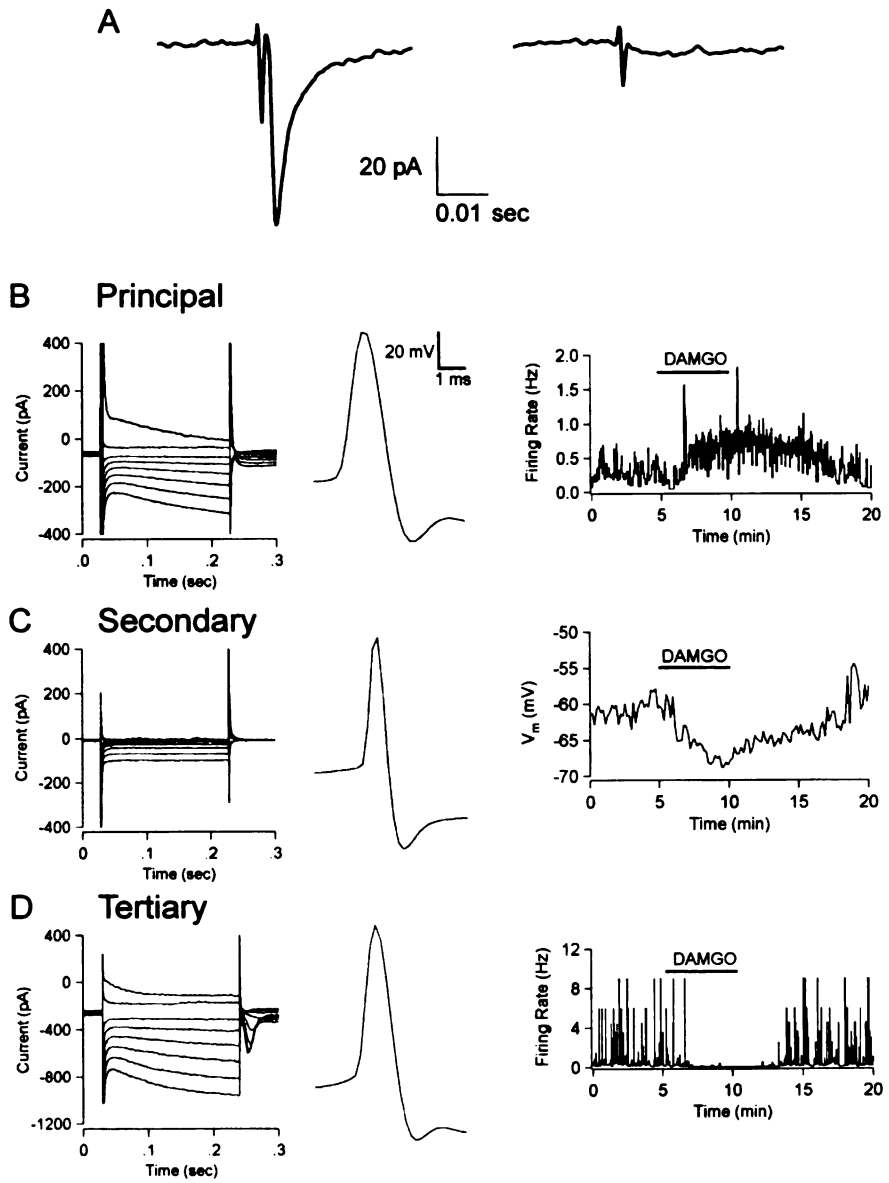
A
20 pA
0.01 sec
B Principal
Current (pA)
Time (sec)
20 mV
1 ms
Firing Rate (Hz)
DAMGO
Time (min)
C Secondary
Current (pA)
Time (sec)
V_m (mV)
DAMGO
Time (min)
D Tertiary
Current (pA)
Time (sec)
Firing Rate (Hz)
DAMGO
Time (min)

Figure 3-1

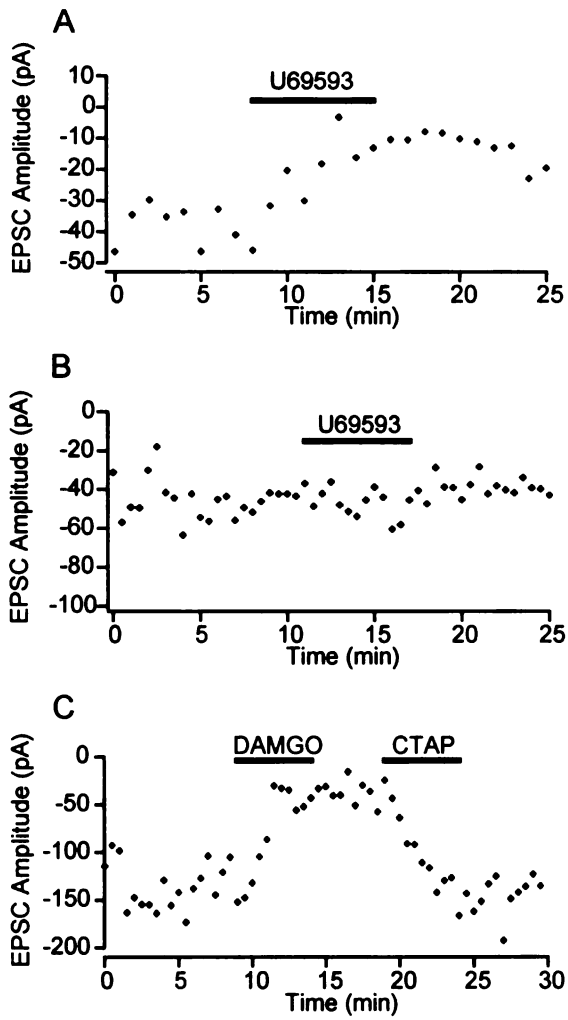
A
EPSC Amplitude (pA)
10
0
-10
-20
-30
-40
-50
U69593
0
5
10
15
20
25
Time (min)
B
EPSC Amplitude (pA)
0
-20
-40
-60
-80
-100
U69593
0
5
10
15
20
25
Time (min)
C
EPSC Amplitude (pA)
0
-50
-100
-150
-200
DAMGO
CTAP
0
5
10
15
20
25
30
Time (min)

Figure 3-2

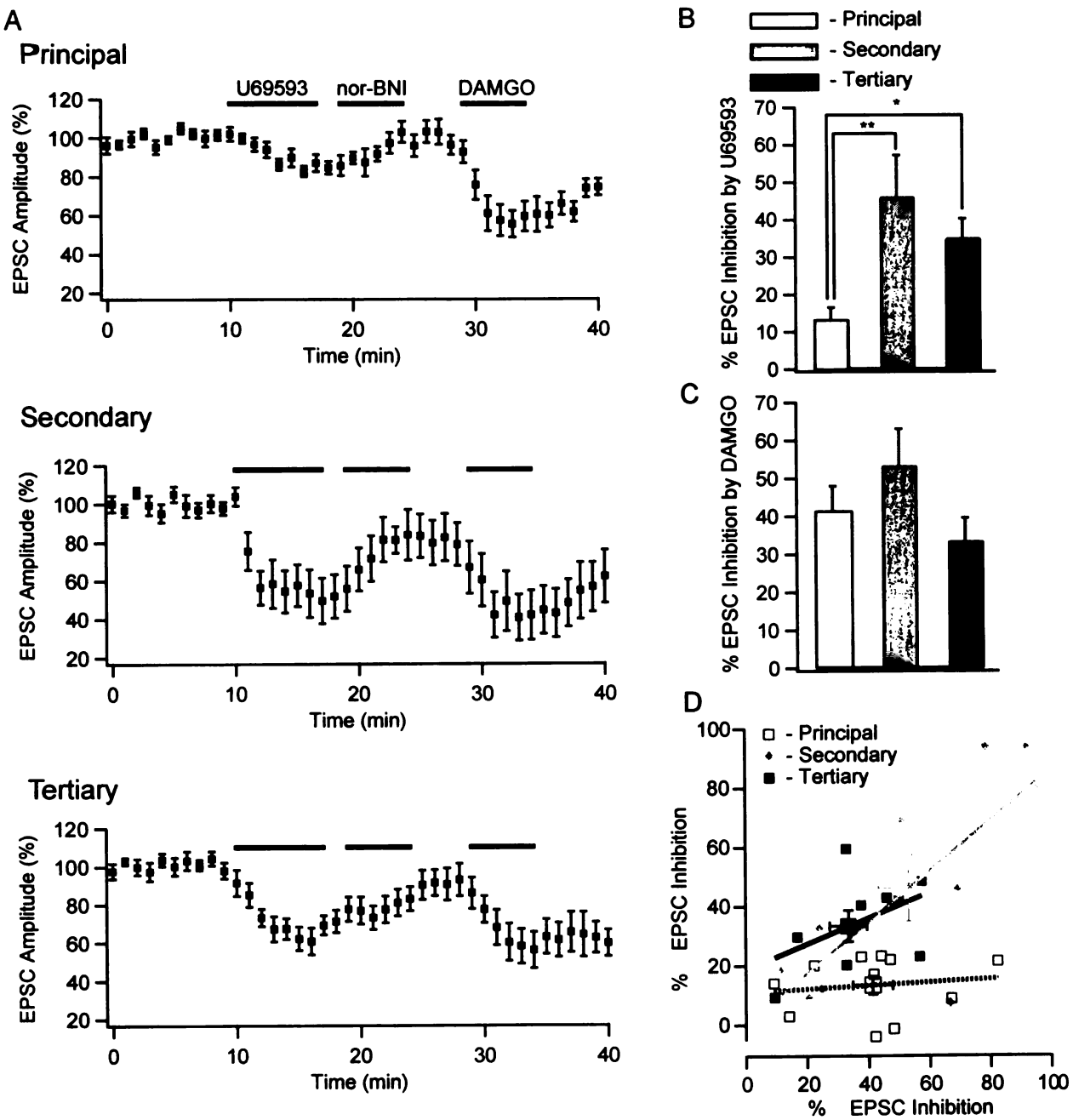
A
Principal
U69593
nor-BNI
DAMGO
EPSC Amplitude (%)
Time (min)
Secondary
Tertiary
B
- Principal
- Secondary
- Tertiary
% EPSC Inhibition by U69593
C
% EPSC Inhibition by DAMGO
D
% EPSC Inhibition
% EPSC Inhibition

Figure 3-3

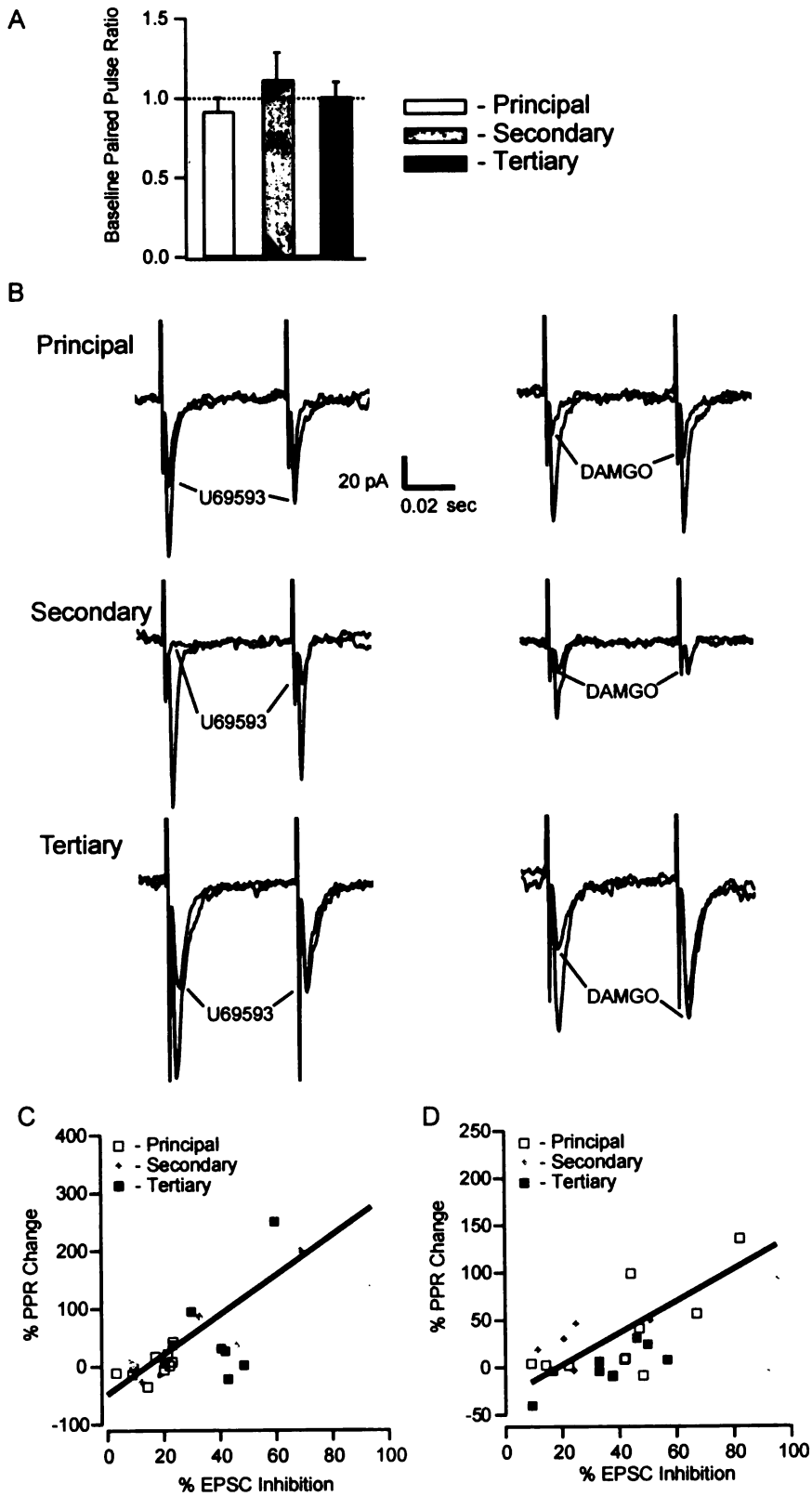
A
Baseline Paired Pulse Ratio
1.5
1.0
0.5
0.0
- Principal
- Secondary
- Tertiary
B
Principal
U69593
20 pA
0.02 sec
DAMGO
Secondary
U69593
DAMGO
Tertiary
U69593
DAMGO
C
- Principal
- Secondary
- Tertiary
% PPR Change
400
300
200
100
0
-100
0
20
40
60
80
100
% EPSC Inhibition
D
- Principal
- Secondary
- Tertiary
% PPR Change
250
200
150
100
50
0
-50
0
20
40
60
80
100
% EPSC Inhibition

Figure 3-4

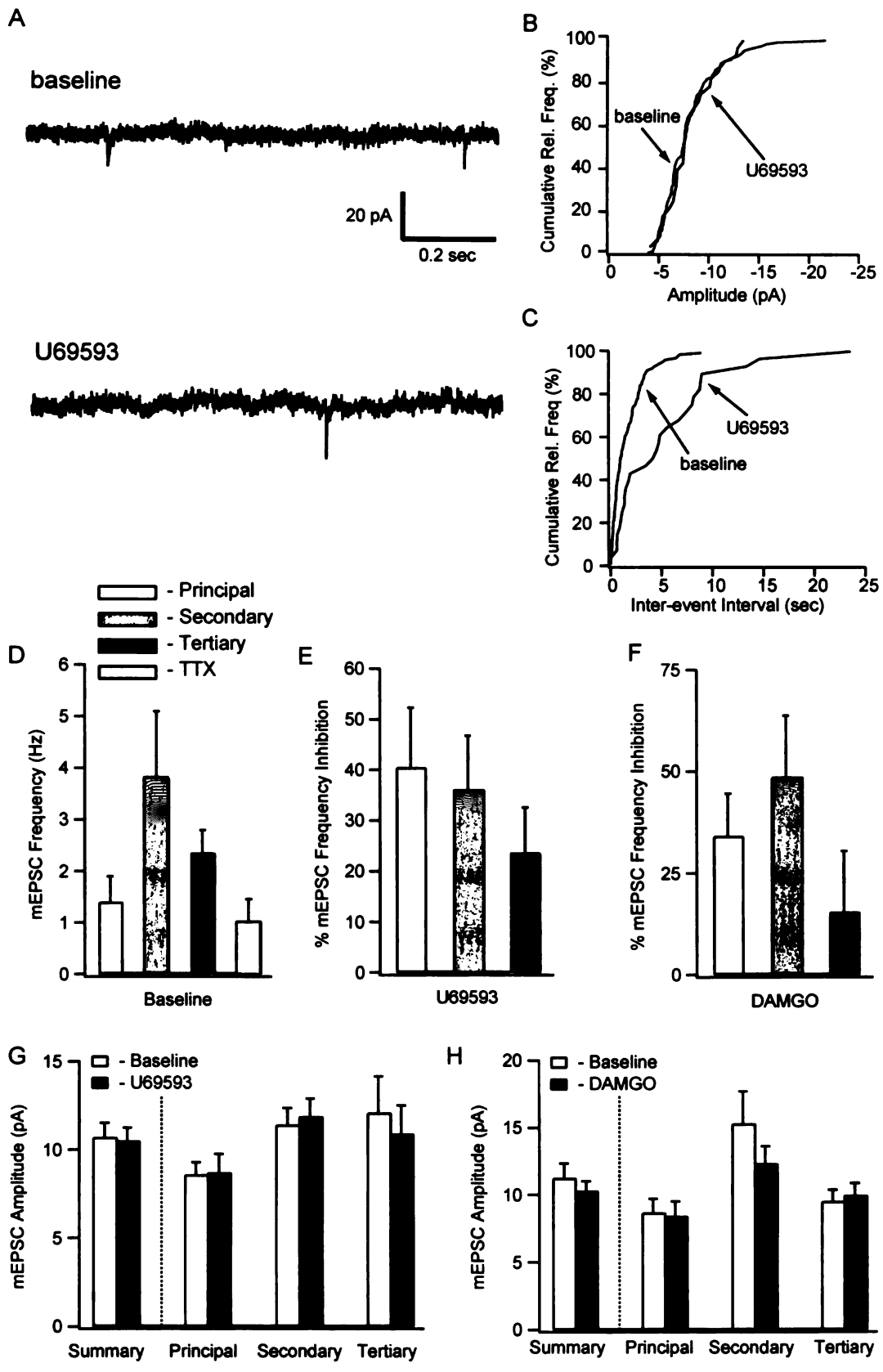
A
baseline
20 pA
0.2 sec
U69593
B
Cumulative Rel. Freq. (%)
baseline
U69593
Amplitude (pA)
C
Cumulative Rel. Freq (%)
U69593
baseline
Inter-event Interval (sec)
- Principal
- Secondary
- Tertiary
- TTX
D
mEPSC Frequency (Hz)
Baseline
E
% mEPSC Frequency Inhibition
U69593
F
% mEPSC Frequency Inhibition
DAMGO
G
- Baseline
- U69593
mEPSC Amplitude (pA)
Summary
Principal
Secondary
Tertiary
H
- Baseline
- DAMGO
mEPSC Amplitude (pA)
Summary
Principal
Secondary
Tertiary

Figure 3-5

Chapter 4

Characterizing VTA neurons *in vitro*

Introduction

Midbrain dopaminergic neurons are widely studied because of their involvement in motivation and learning, as well as in addiction, schizophrenia, and Parkinson's Syndrome. Determining the physiological function of dopaminergic neurons requires electrophysiological recordings *in vivo*. Because such neurons are intermixed with other types of neurons in midbrain areas such as the ventral tegmental area (VTA), criteria for distinguishing dopaminergic and non-dopaminergic neurons is essential to the interpretation of *in vivo* extracellular recordings. Such criteria are obviously of great importance when hypotheses are formulated about what dopamine neurons contribute to behavior.

The earliest attempts to identify dopaminergic neurons in the midbrain focused on the substantia nigra pars compacta (SNC), which contains the A9 group of dopaminergic neurons. In early *in vitro* studies of the SNC, two populations of neurons with distinct characteristics were discerned through electrophysiological recordings and immunohistochemistry (Grace and Onn, 1989; Yung et al., 1991; Richards et al., 1997). These characteristics included action potential length, resting membrane potential, action potential threshold, firing rate, the presence of a hyperpolarization-activated non-specific cation current (I_h), and inhibition by dopamine or a μ opioid (MOP) receptor agonist.

The neighboring VTA is also a major source of dopamine, although it contains a higher percentage of non-dopaminergic neurons than the SNC, and overall is more

heterogeneous than the SNC in its connections and neuronal types. The prevailing opinion is that most of the non-dopaminergic neurons in the VTA have gamma-aminobutyric acid (GABA) as their neurotransmitter, and these neurons project to many of the same brain regions as the dopamine neurons. Non-dopaminergic VTA neurons also provide the major VTA input to the habenula and the hippocampus (Swanson, 1982). A subset of GABAergic neurons in the VTA have been postulated to be local interneurons. Electrophysiological characterization of the VTA *in vitro* followed that of the SNC. Essentially the same electrophysiological distinctions observed in the SNC could be made among VTA neurons (Johnson and North, 1992a).

However, the tools used as electrophysiological and pharmacological markers for dopamine content in the SNC appear to be less reliable predictors for VTA neurons. In Johnson and North's (1992a) electrophysiological characterization of 2 types of neurons in the VTA, only 3 of 5 neurons with the properties attributed to dopaminergic neurons co-stained for tyrosine hydroxylase (TH), an immunohistochemical marker of dopamine neurons (Pickel et al., 1976, 1977). Studies in which more neurons were immunohistochemically processed provide more robust evidence that the properties that reliably define dopaminergic neurons in the SNC are not consistently predictive of dopamine content in VTA neurons. Specifically, the most common properties used for cell type identification, namely the presence or absence of an I_h current, action potential duration, and pharmacological sensitivity to dopamine or MOP receptor agonists, have proven unreliable. While those neurons in the VTA that lack an I_h current are consistently TH negative, many of those exhibiting the current are not dopaminergic. For instance, Cameron, Wessendorf, and Williams (1997) identified a population of large

neurons that exhibit an I_h current and are hyperpolarized by MOP receptor agonists and serotonin. They termed these neurons "tertiary cells." Only forty percent of tertiary neurons were dopaminergic. These results indicate that there are subsets of 1) non-dopaminergic neurons with an I_h current and 2) dopaminergic neurons that are inhibited by MOP receptor agonists in the VTA. Evidence for non-dopaminergic neurons in the VTA with an I_h current was also demonstrated by Jones and Kauer (1999) who reported TH staining in only 2/3 of their I_h neurons. We similarly reported TH staining in just over 2/3 of neurons with an I_h current (Margolis et al., 2003). Together, these studies suggest that testing for an I_h current and MOP sensitivity is not an adequate indicator of dopaminergic content.

These results raise the question: is there a marker, either in lieu of or conjunction with I_h, that can be observed during electrophysiological recording and used as an accurate predictor of dopamine content in the VTA? Statistical analysis of the *in vitro* data leads to the conclusion that in neurons expressing an I_h current, the presence of a postsynaptic hyperpolarization by a MOP receptor agonist or serotonin makes the neuron less likely to be dopaminergic. Interestingly, Di Mascio and Esposito (1997) demonstrated *in vivo* that VTA neurons that are inhibited following systemic administration of serotonin reuptake blockers have a different inter-spike interval density power spectrum from those neurons that are excited in this paradigm. These different neuron populations do not necessarily correspond to principal and tertiary neurons due to differences between *in vivo* and *in vitro* conditions. However, this study does suggest a direction of analysis for *in vitro* data since most VTA neurons are firing spontaneously in the slice.

The *in vitro* and immunohistochemical results reviewed here highlight the shortcomings of current electrophysiological and pharmacological cell type identification methods. In an attempt to identify a more reliable method for identifying dopaminergic neurons in vitro, a number of anatomical, pharmacological, and electrophysiological properties of I_h expressing neurons are analyzed here. Properties including cell shape and location, κ and μ sensitivity, I_h size, baseline firing rate, inter-spike interval variability, and inter-spike interval density power spectrum were compared across neurons.

Experimental Procedures

Slice Preparation and Electrophysiology

20-36 day old male Sprague-Dawley rats were anesthetized with isoflurane, and the brains were removed. Horizontal brain slices (150-250 μm thick) containing the VTA were prepared using a vibratome (Leica Instruments, Germany). Slices were submerged in Ringer solution containing (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 35°C for at least 1 hour.

Individual slices were visualized under a Zeiss Axioskop with differential interference contrast optics and infrared illumination. Whole cell patch clamp recordings were made at 31°C using 2.5-4 MΩ pipettes containing (in mM): 123 K-gluconate, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 MgATP, and 0.3 Na_3GTP (pH 7.2, osmolarity adjusted to 275). Biocytin (0.1%) was added to the internal solution for experiments in which cells were filled for later TH staining.

Recordings were made using an Axopatch 1-D (Axon Instruments, Union City, CA), filtered at 2 kHz and collected at 5 kHz using IGOR Pro (Wavemetrics, Lake Oswego, OR). Liquid junction potentials were not corrected during current- or voltage-clamp recordings. I_h currents were recorded by voltage clamping cells and stepping from -60 to -40, -50, -70, -80, -90, -100, -110, and -120 mV. Cells were recorded in current clamp mode (I=0) for experiments measuring spontaneous firing rates. In some experiments, 500 nM TTX was added to the bath solution to block synaptic transmission after a stable 10 minute baseline was observed, and U69593 and DAMGO were subsequently added to this TTX solution. Current-voltage data was collected in voltage clamp by stepping from a holding potential of -60 mV to -40 mV and ramping down to -140 mV over a 2 second interval. Dose response data was collected with repeated applications of increasing doses of U69593 in each cell, and is reported as the percent of the inhibition produced by a maximal dose of 5 μM in each responding cell.

All drugs were applied by bath perfusion. Stock solutions were made and diluted in Ringer immediately prior to application. U69593 stock was diluted in 50% EtOH to a concentration of 1 mM; nor-Binaltorphimine (nor-BNI; 10 mM), and [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-Enkephalin (DAMGO; 1 mM), were diluted in H_2O; tetrodotoxin (TTX; 5mM) was diluted in DMSO. Agonists, antagonists, ATP, and GTP were obtained from Sigma Chemical (St. Louis, MO) or Tocris (Ballwin, MO).

Data Analysis

All the analyses done here were completed on data collected for previous experiments investigating the postsynaptic effects of KOP receptor agonists in the VTA. All of the electrophysiological analyses were limited to spontaneously active neurons that were

firing stably and consistently for the duration of the experiment in an attempt to limit comparisons to equivalently healthy neurons.

For data analysis, instantaneous firing rate was computed as the inverse of the inter-spike interval after each action potential. Results are presented as mean ± SEM where appropriate. For each cell, the statistical significance of drug effects was tested with the paired student t-test, comparing the last 4 minutes of baseline to the last 4 minutes of drug application. The membrane potential reported here is the initial potential measured immediately after breaking the seal to a cell. The action potential width was taken as the width at half the height of the action potential. The action potential height was measured as the potential difference between the point of minimum positive slope prior to the action potential and the recorded peak potential. All analyses of spontaneous activity were done on 501 spikes under baseline conditions prior to drug application.

The Ψ factor, a measure of chaos in the series of interspike intervals (ISI), was calculated as previously described and summarized below (Di Mascio and Esposito, 1997; Di Mascio et al., 1999). The Ψ factor is,

$$\Psi(\Delta t_j(n)) = \sum_{k=0}^{N-1} \frac{|F(k)|}{F(0)} \text{ where}$$

$\Delta t_j(n)$ is the series of interspike intervals and N=501, the number of spikes analyzed here. F(x) is the fast fourier transform function,

$$F(k) = \sum_{n=1}^{N} \Delta t_j(n) \cos\left(\frac{2\pi nk}{N}\right) - i\Delta t_j(n) \sin\left(\frac{2\pi nk}{N}\right)$$

and therefore,

$$F(0) = \sum_{n=1}^{N} \Delta t_j(n).$$

Immunohistochemistry

Immediately after recording, slices were fixed for 2 hours in 4% formaldehyde. Slices were pre-blocked for 2 hours in PBS with 0.2% Triton X-100, 0.2% BSA and 5% normal goat serum. Rabbit anti-tyrosine hydroxylase antibody (1:100) was then added and the slices agitated at 4° C for 48 hours. Finally, slices were agitated with FITC anti-rabbit secondary antibody (1:100) and Texas Red avidin (11.0 microL /mL) at 4° C overnight. Cells were visualized with a Zeiss LSM 510 META microscope.

Biocytin, BSA, and normal goat serum were obtained from Sigma Chemical (St. Louis, MO). Texas red avidin was obtained from Jackson Immunoresearch (West Grove, PA), and rabbit anti-tyrosine hydroxylase antibody and FITC anti-rabbit secondary antibody were obtained from Chemicon (Temecula, CA).

Results

Whole cell patch clamp recordings were made of neurons in the VTA. All records of spontaneous activity were made in current clamp with I=0. In all cases, neurons were tested for the presence of an I_h current and postsynaptic inhibition by both MOP and kappa opioids (KOP), and these results were reported previously (Margolis et al., 2003). The baseline properties of the neurons in that study are analyzed here for possible differences between cell types. These data were chosen in order to avoid sampling bias that might arise from collecting new data specifically for these analyses. The populations of I_h neurons that were TH(+) and TH(-) were completely overlapping in their anatomical location (Fig. 4-1).

The electrophysiological analyses described below were limited to neurons firing spontaneously with stable and persistent firing rates in order to avoid artifacts that may be caused by poor neuron or slice health. Six TH(+) and 3 TH(-) neurons fit this criterion. Among these neurons, there was no difference between initial membrane potentials (Table 4-2). The I_h current in TH(-) neurons was significantly larger than that of TH(+) neurons ($P<0.01$; Fig. 4-2; Table 4-2). No difference in I_h shape was observed. The proportion of TH(+) neurons that were spontaneously active was only slightly higher than TH(-) neurons (63.3% vs 50.0%) and this difference was not significant. There was also no difference in action potential (AP) shape, including AP width and height (Table 4-2). No differences were found among TH(+) and TH(-) neurons for other firing properties including interspike interval histogram peak, skew, or standard deviation (Table 4-3).

Sixteen neurons with an I_h current were both pharmacologically tested for postsynaptic KOP and MOP receptor agonist sensitivity and immunohistochemically identified as TH(+) (n=11) or TH(-) (n=5). The KOP receptor agonist (U69593, 1 μM) inhibition was limited to a subset of TH(+) neurons (6/11); no TH(-) neurons were inhibited by U69593. Further, 3 of 11 TH(+) neurons were inhibited by the MOP receptor agonist DAMGO (3 μM). All 3 neurons were also inhibited by U69593.

Because of the low number of neurons that were stably spontanteously active among the immunohistochemically processed cells, additional analyses were carried out for neurons classified electrophysiologically and pharmacologically as principal or tertiary. In these analyses, I_h neurons inhibited by MOP receptor agonists were compared to those not inhibited (tertiary vs principal neurons, respectively). No differences were found in the initial membrane potential, I_h size, baseline firing rate, action potential width, or

action potential height between these groups (Table 4-4). There was a significant difference between the skew of the ISI histogram in these neurons, but not the standard deviation (Fig. 4-3; Table 4-3). This greater deviation from a normal distribution suggests that the pacemaker activity observed in VTA neurons *in vitro* varies in its regularity between principal and tertiary neurons.

Because previous studies found *in vivo* that VTA neurons that are inhibited by serotonin had significantly different density power spectrums from those in neurons not inhibited by serotonin, I tested the hypothesis that this property may also delineate principal neurons from tertiary neurons *in vitro* since tertiary, but not principal neurons are inhibited by serotonin. This measurement, represented by the Ψ factor, is a quantification of the regularity of neuronal firing; a low Ψ factor indicates a very regular firing pattern (ie, little variation in ISI) and a larger factor indicates greater variability, or chaos, in the firing pattern. The Ψ factor was found to be significantly different between principal and tertiary neurons (Fig. 4-4; Table 4-3). The Ψ factor values for these 2 populations have somewhat overlapping distributions. However, it is possible that this overlap is due to the imprecise correlation between this classification method and dopamine content.

Discussion

The analyses of neuronal populations described above suggest a few potential directions for identifying neurons as dopaminergic *in vitro*. This data show that neurons with the largest I_h currents are most likely not dopaminergic. Although the populations of immunohistochemically identified neurons analyzed here are too small to yield differences in firing properties of dopaminergic and non-dopaminergic neurons with an

I_h, it is possible that the findings in the principal and tertiary spontaneous activity data, specifically the skew and density power spectrum of the distribution of ISIs, may similarly vary between dopaminergic and non-dopaminergic neurons. These characteristics may provide more accurate cell identification methods than current electrophysiological measures.

A well-defined method for classifying neurons in the VTA is not yet available. In studies where all I_h neurons express the property or sensitivity being tested, it is reasonable to conclude that the subset of dopaminergic neurons that are included in the I_h neurons. Unfortunately, many of these studies fail to recognize that by classifying neurons by I_h alone, 1/3 of the observations were made in non-dopaminergic VTA neurons. Classifying neurons as principal, secondary, and tertiary based on I_h current and MOP receptor agonist inhibition allows for more accurate identification of dopaminergic neurons; only about 20% of principal neurons are TH(-). However, since approximately 40% of tertiary neurons are dopaminergic, studying principal neurons alone excludes a non-trivial population of dopaminergic neurons.

The differences between the properties of firing patterns in principal and tertiary neurons found here suggest that there are other properties that differ between these 2 neuron types. Additionally, while the immunohistochemically identified populations are too small here to yield significant differences in these properties, it is possible that more data may lead to similar observations between TH(+) and TH(-) neurons. If this is indeed the case, these analyses of firing properties may prove more accurate than the current electrophysiological and pharmacological tools for identifying dopaminergic neurons.

Pharmacologically, KOP receptor agonist inhibition is limited to dopaminergic neurons. This observation is interesting in this context because it makes KOP inhibition an accurate predictor of dopamine content. Only about half of the dopaminergic neurons exhibit this KOP-mediated inhibition, and therefore this measure is inadequate for identifying all dopamine neurons. However, one hypothesis is that KOP-inhibited dopaminergic neurons project to only certain VTA targets. If this is indeed the case, KOP sensitivity may prove useful as an identification method, depending on the hypothesis driving the experimental design.

In many cases, postsynaptic MOP inhibition has been used to identify VTA neurons that are most likely not dopaminergic. Indeed, regardless of whether or not a neuron has an I_h current, it is most likely non-dopaminergic if it is inhibited by a MOP receptor agonist. It is interesting to note that while the MOP receptor agonist DAMGO directly inhibits some TH(+) neurons, this only occurred in neurons that are also inhibited by the KOP receptor agonist, suggesting that there is some more complex correlation between MOP inhibition and dopamine content in the VTA.

The I_h and KOP inhibition observations presented here highlight how classifying neurons according to I_h current and MOP sensitivity can obscure differences between dopaminergic and non-dopaminergic neurons in the VTA. Based on principal and tertiary neuron data, the conclusion would be drawn that neurons with an I_h in the VTA all have similarly sized I_h currents. However, the immunohistochemical data here reveals that neurons with the largest I_h currents are in fact not dopaminergic. In the case of the postsynaptic KOP sensitivity, since both principal and tertiary neurons exhibit a KOP induced inhibition, the necessary conclusion would be that both dopaminergic and non-

dopaminergic neurons in the VTA are inhibited by KOP receptor agonists. Yet the immunohistochemistry shows that only dopaminergic neurons are inhibited by KOP receptor agonists. These issues highlight the need for more careful classification of VTA neurons during both *in vitro* and *in vivo* experiments.

The electrophysiological and pharmacological differences observed here suggest directions for future study. Because of the small population of immunohistochemically identified neurons currently available for this analysis and other anatomical studies that need to be done, the usefulness of these measures as tools for dopaminergic neuron identification is not yet clear. It is imperative that these properties show little overlap in larger samples and stability across investigators in order to be considered accurate predictive tools. The observations reported here do suggest directions for more rigorous investigation, and therefore preserve the possibility of an electrophysiological tool for the identification of dopaminergic neurons in the VTA.

Table 4-1. Neuron morphology of dopaminergic (TH(+)) and non-dopaminergic (TH(-)) neurons in the VTA.

*ANOVA, $p<0.05$

**ANOVA, $p<0.01$

	Fusiform	Oval	Pyramidal	Round
TH (+)	7	2	1	1
TH (-)	7	2	0	1

Table 4-2. Electrophysiological properties of dopaminergic (TH(+)) and non-dopaminergic (TH(-)) neurons with an I_h current in the VTA.

*ANOVA, $p<0.05$

**ANOVA, $p<0.01$

	Membrane Potential (mV)	I_h size (pA)**	Firing Rate (Hz)	Action Potential Width (msec)	Action Potential Height (pA)
TH (+) (n=6)	44 +/- 1	110 +/- 20	1.1 +/- .3	1.6 +/- .2	68 +/- 9
TH (-) (n=3)	46 +/- 2	290 +/- 40	1.8+/- .7	1.1 +/- .1	63 +/- 4

Table 4-3. Firing properties of dopaminergic (TH(+)) and non-dopaminergic (TH(-)) neurons with an I_h current in the VTA.

*ANOVA, $p<0.05$

**ANOVA, $p<0.01$

	ISI Histogram Peak (msec)	ISI Std Deviation	ISI Skew	Ψ factor
TH (+) (n=6)	550 +/- 80	1200 +/- 500	5 +/- 1	9 +/- 1
TH (-) (n=3)	500 +/- 100	600 +/- 300	6 +/- 3	11 +/- 2

Table 4-4. Electrophysiological properties of principal and tertiary neurons with an I_h current in the VTA.

*ANOVA, $p<0.05$

**ANOVA, $p<0.01$

	Membrane Potential (mV)	I_h size (pA)	Firing Rate (Hz)	Action Potential Width (msec)	Action Potential Height (pA)
Principal (n=7)	-46 +/- 2	470 +/- 80	.9 +/- .1	1.3 +/- .2	70 +/- 10
Tertiary (n=9)	-48 +/- 3	320 +/- 70	1.0 +/- .1	1.3 +/- .1	80 +/- 10

Table 4-5. Firing properties of principal and tertiary neurons with an I_h current in the VTA.

*ANOVA, $p<0.05$

**ANOVA, $p<0.01$

	ISI Histogram Peak (msec)	ISI Std Deviation	ISI Skew**	Ψ factor*
Principal (n=7)	1000 +/- 100	700 +/- 200	4.5 +/- 0.6	6.0 +/- 0.9
Tertiary (n=9)	700 +/- 100	700 +/- 100	9 +/- 1	10 +/- 2

Figure Legends

Figure 4-1. Dopaminergic and non-dopaminergic neurons in the VTA are anatomically overlapping. Represented here in half of a horizontal brain slice (right is medial, top is rostral) are the locations of neurons in the VTA that expressed an I_h current and were immunohistochemically processed for TH immunoreactivity. TH positivity indicates dopamine content.

Figure 4-2. Neurons with large I_h currents are not dopaminergic. Among I_h-expressing neurons, TH negative neurons (darker bars) have significantly larger I_h currents than TH positive neurons (ANOVA, $p<0.01$).

Figure 4-3. The skew of the interspike interval of spontaneously active neurons is smaller in non-dopaminergic neurons. Example histograms of 500 ISIs of principal (A) and tertiary (B) neurons show the longer intervals present in principal neurons that account for the larger ISI skews in principal neurons. C, the summary histogram of principal (light bars) and tertiary (dark bars) neuron ISI skews shows overlapping but significantly different (ANOVA, $p<0.01$) populations.

Figure 4-4. The spontaneous activity of principal neurons is more chaotic than that of tertiary neurons. The Ψ factor, a measure of the density power spectrum of ISIs, is smaller among principal neurons (light bars) than tertiary neurons (dark bars), indicating more regular spontaneous activity in principal neurons (ANOVA, $p<0.05$)

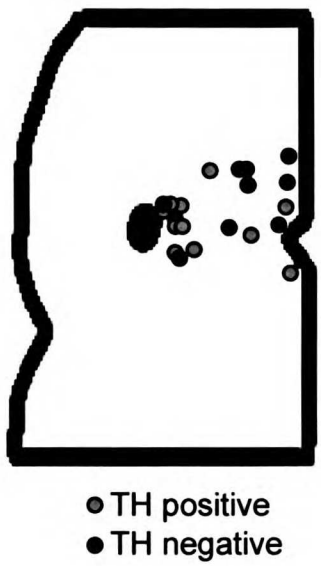

Figure 4-1

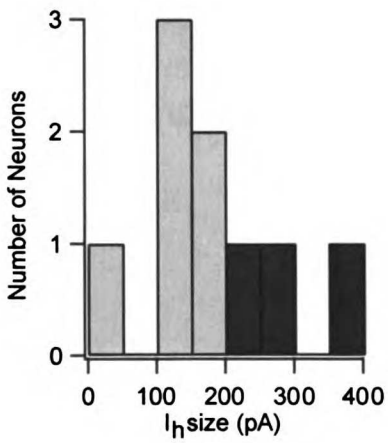

Figure 4-2

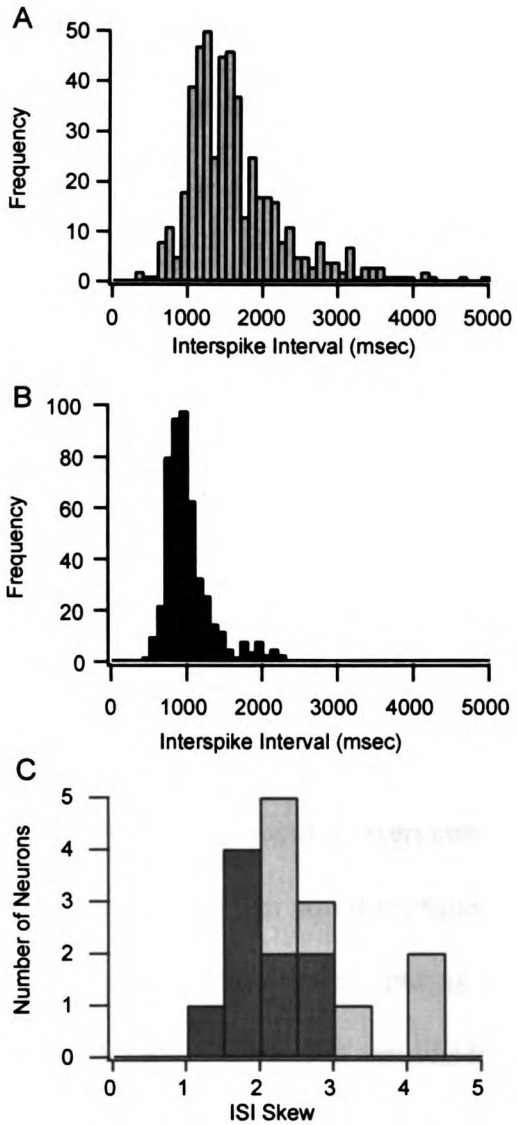

Figure 4-3

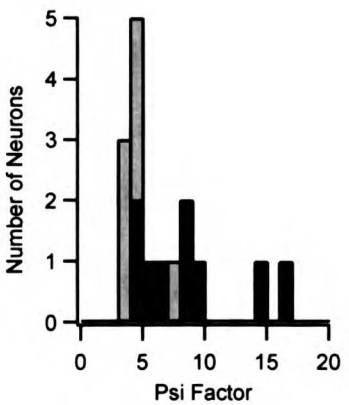

Figure 4-4

Chapter 5

Discussion

Summary of Findings

In the work presented in this thesis, I demonstrated both pre- and postsynaptic actions of κ opioid (KOP) receptor agonists in the ventral tegmental area (VTA). Direct inhibition by KOP receptor agonists is limited to dopaminergic neurons in the VTA, about half of which show this effect (Fig. 5-1). Presynaptically, KOP receptor agonists broadly inhibit glutamate release onto all classes of VTA neurons including non-dopaminergic neurons. Therefore, KOP receptor ligands can modulate a subset of VTA dopaminergic neurons irrespective of input or exert control that is selective for particular afferent inputs to either dopaminergic or non-dopaminergic neurons.

The postsynaptic results provide a simple opposing mechanism for μ opioid (MOP) and KOP receptors in the VTA. A number of neurons in the postsynaptic study exhibited both inhibition by KOP and disinhibition by MOP receptor agonists (Fig. 5-2). This opposition offers a simple explanation for the KOP-induced place aversion and MOP-induced place preference that result from injections of these drugs directly into the VTA.

Interestingly, the presynaptic actions on glutamate terminals of KOP and MOP receptor agonists are grossly similar. These findings raise questions of whether or not these receptors are found on the same terminals, and, if not, whether the neurons that give rise to the KOP- and MOP-sensitive terminals are located in the same brain region.

The similarities between KOP and MOP presynaptic effects, and the subset of dopaminergic neurons that were inhibited by both KOP and MOP receptor agonists

contrasts with the motif of functional KOP and MOP opposition on dopaminergic neurons in the VTA. This is not entirely unexpected given that there are some behaviors, such as feeding and intracranial self stimulation in the hypothalamus, that are similarly affected by KOP and MOP receptor agonists in the VTA. It is possible that these behaviors are mediated by the subset of dopaminergic neurons that are inhibited by both KOP and MOP receptor agonists. Alternatively, modulation of VTA glutamatergic inputs may account for the similar behavioral effects of KOP and MOP receptor agonists since the presynaptic inhibitions are also similar. Because of the variety of synaptic effects of KOP on different classes of VTA neuron it is essential to know whether the neurons demonstrating KOP postsynaptic inhibition have projection targets that are distinct from those that do not exhibit this effect. Similarly, it will be interesting to know the origin of KOP regulated afferent terminals and under what behavioral conditions they are activated.

Finally, the data presented here provide evidence that current electrophysiological and pharmacological markers are insufficient to delineate dopaminergic neurons from non-dopaminergic neurons in the VTA. Over 30% of the neurons expressing an I_h current, the classic electrophysiological marker for dopamine neurons, are non-dopaminergic (TH(-)). Further, half of the tertiary neurons (MOP sensitive inhibited with an I_h) that have formerly been identified as not dopaminergic (Cameron et al., 1997) were TH (+). However, the analyses included in chapter 4 provide evidence that properties other than pharmacological sensitivity to MOP receptor agonists and serotonin differentiate tertiary neurons from principal neurons. That the Ψ factor of the baseline interspike interval series of principal and tertiary neurons is different both *in vivo* and *in*

vitro suggests that this firing pattern property is due to something intrinsic in the VTA. Even more importantly, this measurement can be used as a tool for drawing parallels between *in vitro* and *in vivo* observations in subsets of VTA neurons that have been shown to be pharmacologically different but are usually not differentiated during experiments. The Ψ factor analysis is particularly useful since it does not require any electrical or pharmacological manipulation of the system. Two challenges in using a measurement that is based on firing patterns are that the neurons that are to be analyzed must be firing spontaneously, and that variations in the health of the tissue due to experimental methods could influence the regularity of a neuron's firing. The extended analyses presented in chapter 4 suggest directions for further study for more reliable characteristics for indirectly determining dopaminergic content.

Future Work

The studies presented here all suggest both the need and the most fruitful directions for additional investigations of the VTA. The first order of business is to establish a classification scheme for VTA neurons that leads reliable predictions of their properties within subgroups. Several approaches have been used: dopaminergic versus non-dopaminergic, I_h versus non-I_h, principal/secondary/tertiary, KOP or MOP responsive versus non-responsive. With the exception of secondary cells which are non-dopaminergic, directly MOP responsive, and non-I_h expressing, each of these approaches has led to subgroups that are heterogeneous. Clearly, other criteria are needed to provide homogeneous neuronal groupings.

Along these lines, an obvious question is whether the observed KOP and MOP sensitivities segregate selectively to groups of VTA neurons that project to different brain

regions. This question can be addressed by injecting a retrograde marker into VTA projection targets in the intact animal, allowing the dye to be transported back to the VTA, and label neurons that can then be identified and recorded from *in vitro*. Previous anatomical studies have shown that individual VTA neurons do not project to more than one brain region and that afferent inputs from a given brain region are differentially distributed among different VTA neurons, i.e. there are subpopulations of VTA neurons that participate in different neural circuits (Swanson, 1982; Carr and Sesack, 2000). Therefore, it is distinctly possible that the subpopulations of neurons inhibited by KOP receptor agonists, MOP receptor agonists, both, or neither, project to different brain regions (Fig. 5-3). For instance, since microdialysis experiments show an increase in NAc dopamine in response to VTA injections of MOP receptor agonists but no change in response to VTA injections of KOP receptor agonists (Spanagel et al., 1992; Devine et al., 1993b), it is likely that dopaminergic neurons that project to the NAc are not directly inhibited by MOP or KOP receptor agonists. Since the amygdala plays an important role in modulating the affective salience of sensory cues (e. g. Fudge and Emiliano, 2003), it may be the target of dopaminergic neurons exhibiting opposing MOP and KOP actions. Both feeding, which is powerfully modulated by the hypothalamus, and intracranial self stimulation of the hypothalamus are similarly affected by KOP and MOP opioid injections into the VTA (Mucha and Herz, 1985; Hamilton and Bozarth, 1988; Singh and Desiraju, 1988; Singh et al., 1994; Lamonte et al., 2002); therefore it is possible that dopaminergic neurons that are inhibited by both KOP and MOP receptor agonists project to the hypothalamus. Another possibility is that dopaminergic projections to the prefrontal cortex are inhibited by both MOP and KOP receptor agonists. Dopaminergic

VTA projections to the mPFC are involved in attention (e. g. Viggiano et al., 2003), and therefore these projections are most likely similarly engaged by VTA projections regardless of the affective quality of a drug's effect.

Similar anatomical information would also likely enhance our understanding of the functional significance of the presynaptic KOP receptor agonist effects. In this case, it would be interesting to know if 1) the projection target of a neuron determines the magnitude of EPSC inhibition by KOP agonists and 2) the glutamatergic fibers from different brain regions are differentially inhibited by KOP and MOP receptor agonists. Determining the role that the projection target plays in the presynaptic glutamate inhibition could be investigated in a manner similar to that described for the postsynaptic study. The second question could be addressed by individually lesioning the sources of glutamate afferents to the VTA prior to making *in vitro* recordings to determine if loss of input from a single area results in a significant drop in the presynaptic effect of a KOP agonist on glutamatergic transmission. One challenge in these experiments is that glutamatergic fibers that arise from different brain regions may not all be stimulated to a similar extent given the placement of the stimulating electrode in the slice, and this could lead to difficulties in interpreting differences between recordings from intact and lesioned animals.

Repeating both the post- and presynaptic studies in drug-exposed animals is another possible direction for future study. There is anatomical evidence that KOP receptor expression levels in the VTA change following exposure to drugs of abuse. Exposure to both cocaine and alcohol greatly decrease KOP receptor mRNA in the VTA (Rosin et al., 1999). The functional ramifications of this change have not yet been measured. While

the postsynaptic effects would be of principal interest because of the MOP and KOP opposition, changes in presynaptic modulation of the VTA could also contribute significantly to the functioning of the VTA following drug exposure. Recent studies suggest that presynaptic expression of opioid receptors is greatly altered by drug exposure (Bie and Pan, 2003; Christie and Hack, 2003). It is possible that presynaptic KOP receptor expression in the VTA is similarly state dependent, and that a change in expression levels produces an imbalance in KOP receptor agonist influence over VTA function. Therefore, it would be useful to measure both pre- and postsynaptic KOP receptor agonist functional potency in drug-exposed animals.

Methodologically, an important question raised by the analyses in Chapter 4 is whether or not the differences found between principal and tertiary neurons correspond to differences between TH(+) and TH(-) neurons. Further investigations should also optimize the measures based on interspike interval data that prove useful as tools. This optimization includes determining the minimum number of spikes that must be analyzed in order to maintain reliable separation between neuron types, and whether or not using a combination of measures significantly increases the reliability of cell type identification. Such a methodological advance would not only make *in vitro* neuron identification easier, but could provide a new tool for *in vivo* cell type identification.

An *in vitro* study suggested by the literature is a comparison of MOP and δ opioid (DOP) effects in the VTA. The available behavioral data suggests that MOP and DOP receptor activation in the VTA have similar effects. However, a DOP system that is simply a weaker version of the MOP system seems unusually redundant for biological systems. To elucidate the differences between these systems, experiments similar to

those completed here with KOP and MOP would need to be completed. Additionally, since MOP and DOP receptors in the VTA are differentially expressed following amphetamine exposure (Magendzo and Bustos, 2003) and, in other parts of the brain, DOP is only functionally expressed following opiate exposure (Christie and Hack, 2003), comparing drug-exposed animals to naïve animals would be a useful approach to determining the functional significance of DOP receptor expression.

Addiction and Therapeutic Design

The work completed in this thesis was motivated by the desire to understand how KOP receptor agonists act at the synapse to modulate VTA output in order to relate these actions to the MOP-opposing behavioral effects of KOP receptor agonists. The experimental findings have the potential to inform rational drug design for an addiction therapeutic. Abused drugs have powerful reinforcing properties that motivate users to take them despite the harm they can cause. Inhibiting the activation of the VTA dopaminergic system by drugs of abuse blocks the reinforcing effects of these drugs in many animal models (e. g. Tsuji et al., 1996; Wang et al., 2003). Therefore an ideal strategy for the development of an addiction therapeutic is to focus on molecules that modulate the dopaminergic system.

The KOP receptor is a candidate target for such a therapeutic. KOP receptor agonists specifically inhibit only dopaminergic neurons in the VTA. Drugs of abuse also change the expression levels of KOP receptors and endogenous ligands, suggesting that this system is involved in withdrawal effects, and possibly relapse or craving. Anatomical studies report that KOP receptor mRNA expression in the VTA decreases following cocaine or alcohol administration (Rosin et al., 1999). Therefore, the experiments

suggested above for determining whether this change in message has any functional effects on KOP sensitivity in the VTA are critical for evaluating the KOP receptor as a therapeutic target.

Since KOP receptor agonists can be aversive, only doses below the aversive threshold are practical for human use. Low doses are also desirable since the goal is not to completely inhibit the dopaminergic neurons, but to prevent them from being stimulated by other drugs. Data for the blood plasma levels of the opiate antagonist naltrexone, which is used as a treatment for alcohol addiction (Mason et al., 2002), suggest that a KOP agonist with a similar blood-brain barrier permeability could easily reach the brain at concentrations comparable to those in the middle of the dose-response curve reported in Chapter 2.

The fact that glutamate release in the VTA is also inhibited by KOP receptor agonists provides a second mechanism by which VTA excitation can be limited by a therapeutic. One form of glutamate excitation in the VTA produces a change in the firing pattern of dopaminergic neurons from tonic to phasic. Bursting activity in the VTA is more common in rats that are high responders to drugs, and burst initiation reliably precedes self administration (Marinelli and White, 2000; Phillips et al., 2003). These findings suggest that blocking glutamate-induced bursting in VTA neurons would decrease drug-seeking and consumption. It will be important to determine whether and to what extent the presynaptic effects of KOP agonists contribute to their ability to reduce the CNS reinforcing actions of ethanol and drugs of abuse (Shippenberg and Rea, 1997; Cosgrove and Carroll, 2002).

Since low doses of a KOP receptor agonist would be optimal, a second compound could be chosen to further clamp dopaminergic neuron activity and strengthen the desired effect on drug seeking and consumption. Since MOP receptor activation excites dopaminergic neurons, a MOP receptor selective antagonist that blocks this excitation, in conjunction with a KOP receptor agonist's inhibition, may provide this type of synergistic inhibition of VTA dopaminergic neuron activity. Further, co-administering such drugs at low doses has the advantage of limiting dose-related unwanted side effects of the therapeutic agent while providing synergy only at those neurons that are sensitive to both drug types, such as VTA dopaminergic neurons.

KOP receptors are optimally located for modulation of the motivational and reinforcing signals of the VTA dopaminergic system, and are therefore an excellent candidate target for addiction therapeutic development. KOP receptor activation directly inhibits dopaminergic neurons, as well as inhibits glutamate release to all VTA neurons. The postsynaptic results reported here provide a cellular mechanism for the opposing behavioral effects of KOP and MOP receptor agonists. In addition, the presynaptic actions of MOP and KOP agonists provide a mechanism for opioid control of specific inputs to each VTA cell class. Together, these data provide information essential to our understanding of how the KOP system regulates the motivational effects of both natural rewards and addictive drugs.

Figure Legends

Figure 5-1. KOP receptors are located on a subset of dopaminergic neurons in the VTA. They also presynaptically inhibit glutamate release onto all VTA cell types.

Figure 5-2. A subset of dopaminergic VTA neurons are directly inhibited by KOP receptor activation and disinhibited by MOP receptor activation.

Figure 5-3. Segregation of KOP and MOP receptors on VTA dopaminergic neurons may depend on the projection target of the neurons.

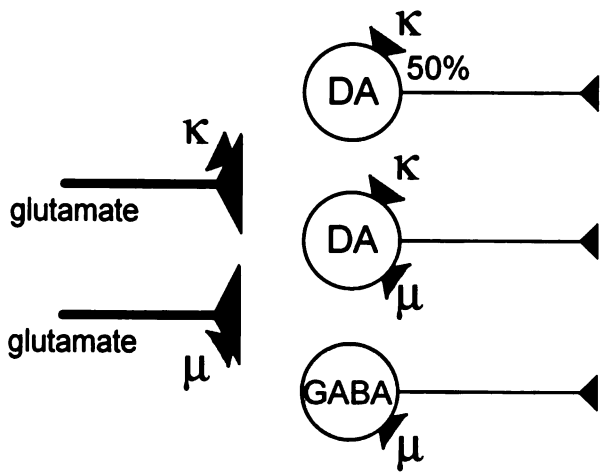

Figure 5-1

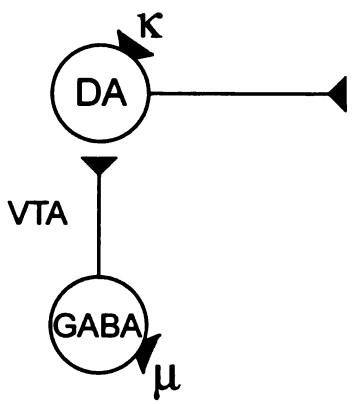

Figure 5-2

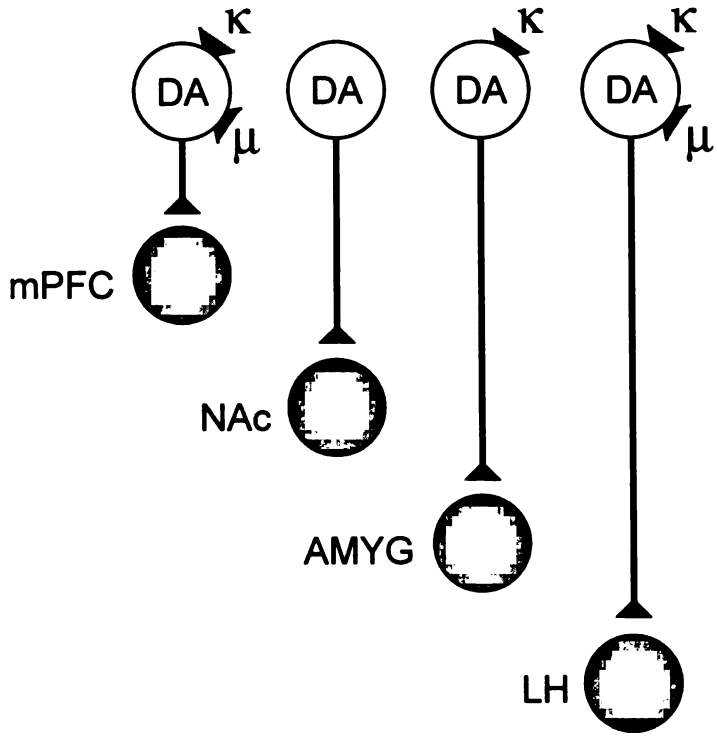

Figure 5-3

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