Hypothalamic modulation of the midbrain dopaminergic system

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Summary

Ventral tegmental area (VTA) dopaminergic (DAergic) and GABAergic neurons are critically involved in mechanisms of reward, reinforcement and emotional arousal. The hypothalamus regulates the homeostatic drive to eat and sends a massive output to the VTA, including projections from neurons containing orexins, the novel neuropeptides, which potently modulate arousal and feeding. Single-unit extracellular and whole-cell patch-clamp recordings, accompanied by the filling of the neuron with biocytin in order to perform post hoc immunostaining, were used to examine the effects of orexins and other hypothalamic neuropeptides on cells in the substantia nigra (SN) and the VTA in vitro. Orexins uniformly excited GABAergic neurons in the SN and the VTA, this effect was blocked by the prior application of a selective protein kinase A inhibitor. A distinct subgroup of GABAergic neurons in the VTA with a slow firing rate (0.8 Hz) was found. In DAergic VTA neurons, orexins caused an increase in firing frequency, burst firing or no change in firing. DAergic neurons in the SN were not affected by orexins. Neurons showing oscillatory firing in response to orexins had smaller afterhyperpolarizations (AHP) than the other groups of dopamine neurons. Single-cell RT-PCR experiments revealed that the calcium binding protein calbindin that is usually present in cells with the smaller AHPs, was only expressed in neurons, which also expressed orexin receptors. All VTA neurons from a recently described group, which express both TH and GAD, expressed orexin receptors and did not express calbindin. In the VTA, in contrast to dorsal raphe, the expression of both orexin receptors was not related to the presence or absence of transient receptor potential canonical channel (TRPC) subunits. Orexins did not affect field potentials in ventral and dorsal striatum. Another stimulator of food intake, neuropeptide Y (NPY) inhibited half of the DAergic and GABAergic neurons in the VTA, whereas the anorectic neuropeptide corticotropin-releasing factor (CRF), which exerts anxiety and arousal, excited a subgroup of DAergic neurons and all tested GABAergic neurons as well. Melanin-concentrating hormone (MCH), agouti-related protein (AGRP), ghrelin, leptin and cocaine and amphetamine-related transcript (CART) did not affect membrane potential or firing rate of the VTA neurons. Substance P (SP) increased the firing rate of the majority of DAergic and all tested GABAergic neurons in VTA. Histamine, a strong wake-promoter, did not affect the firing frequency of DAergic neurons but increased the firing of GABAergic neurons in SN and VTA. This effect was blocked by prior application of the selective H_1 receptor antagonist mepyramine. The novel wake-promoting drug modafinil inhibited DAergic neurons both in VTA and SN. This study shows multiple effects of neuropeptides and monoamines on the mesolimbic system and reflects the complex regulation of arousal and feeding in mammals.

Introduction.

Ventral tegmental area (VTA) dopaminergic and GABAergic neurons are critically involved in brain mechanisms of reward, reinforcement and emotional arousal (Wise and Rompre, 1989). The firing of dopamine neurons in this region is closely correlated with the availability of primary rewards (food, water, sex) (Schultz, 1998). Activation of VTA neurons initiates locomotor activity in order to obtain such primary rewards and this activation is associated with a high level of arousal; compounds which block the dopamine transporter, leading to enhanced dopaminergic tone in target regions, are potent wake-promoting substances (Wisor et al., 2001). The VTA receives a massive input from the lateral hypothalamus (Zahm et al., 2001), including projections from neurons containing the neuropeptides orexins (Fadel and Deutch, 2002) which potently modulate arousal and feeding. Recent evidence has shown that loss of orexin neurons or mutation of the orexin 2 receptor causes the sleep disorder narcolepsy (Willie et al., 2001), which is treated by drugs enhancing dopaminergic tone. The cellular effects of another compound, modafinil, that is also effective in the treatment of narcolepsy, are still unknown. A number of other hypothalamic neuropeptides is also involved in mechanisms of emotional arousal and regulation of feeding and there is a large body of evidence that they could interact with dopaminergic systems. In the first part of my literature review the electrophysiological properties, anatomy and functions of the dopaminergic and GABAergic neurons in substantia nigra and the ventral tegmental area are described, then the hypothalamus, its role in regulation of food intake, and different hypothalamic neuropeptides involved in regulating food intake and arousal. Finally studies regarding the neurotransmitter histamine and the novel waking-inducing drug modafinil in the context of their possible interaction with dopaminergic systems are summarized.

Literature review.

1. Dopaminergic system of the brain.

Cell bodies of dopaminergic (DA) neurons are located in their majority in the ventroanterior midbrain (substantia nigra and ventral tegmental area), in the groups numbered A8 to A10. Their axons project to the dorsal striatum (caudate nucleus and putamen), ventral striatum including nucleus accumbens, and most areas of the neocortex including, prominently, the prefrontal cortex. An additional, smaller dopamine cell group is located in the hypothalamus.

1.1. Electrophysiology and morphology of dopaminergic neurons in VTA and SN.

Electrophysiological properties of DAergic (termed "principal" neurons in some papers) cells in substantia nigra (SN) and the ventral tegmental area (VTA) are extensively described. Intracellular recordings in SN zona compacta in vitro (Lacey et al., 1989) revealed that "principal neurons" fire spontaneous action potentials in the range 1-8 Hz, or are quiescent (33%); they have action potentials greater than 1 msec in duration; show pronounced time-dependent inward rectification - a large "sag" component, which is mediated by the activation of cyclic nucleotide-regulated cation (Ih, HCN) channels, is observed after injection of hyperpolarizing currents. Dopamine inhibits firing and/or hyperpolarizes all principal cells, but mu or delta opioid receptor agonists have no effect. Dopamine and DAergic drugs reduce the firing frequency of DA neurons due to stimulation of D_2-D_3 autoreceptors and to a hyperpolarisation of the membrane produced by an increase in potassium conductance. In zona compacta, in contrast to zona reticulata, 95% of the neurons are dopaminergic. The electrophysiological characteristics of DAergic neurons in SN pars reticulata are identical to those of SN pars compacta, which supports the notion that the dopamine neurons in these two regions are part of the same neuronal population. The magnitude of input resistance and the amplitude of action potentials of DA cells differs in different studies- input resistance range from 80 to 350MOms, the action potentials are generally 50-90mV in amplitude (Richards et al., 1997). The spontaneous, low-frequency, pacemaker activity of these neurons is generated by intrinsic membrane properties. The pacemaker duty cycle appears to be regulated by the interaction of two transmembrane currents: an inward voltage-sensitive pacemaker current (slow depolarization) that depolarizes the membrane to spike threshold, and an outward calcium-activated potassium current responsible for postspike afterhyperpolarization (Grace, 1988). The calcium influx that occurs during the action potential, activates, among others, small-conductance, calcium-activated potassium (SK) channels (Kohler et al., 1996), which in turn generate a large and prolonged AHP that dominates the first part (50-200msec) of the interspike interval during pacemaker discharge and is apamin-sensitive (Shepard and Bunney, 1991). The rebound from the AHP initiates another slow depolarization and completes the pacemaker cycle. Voltage-gated calcium channels play an important role in the AP-mediation of SK channels in DA neurons. They can also be activated by calcium-mobilizing, metabotropic neurotransmitter receptors (Fiorillo and Williams, 1998) or by release of calcium from intracellular calcium stores (Seutin et al., 2000). SK3 mRNA is detected in all TH-positive neurons displaying medium AHPs; the expression of SK1and/or SK2 mRNA is

much lower. There is a significant correlation between I_{AHP} amplitudes and SK3 expression – the lower the SK3 expression, the smaller is I_{AHP}s. The manipulation of SK3 channels in SN affects the firing rate of neurons, although in VTA the discharge activity is not changed after application of an SK3 activator or inhibitor (Wolfart et al., 2001). Activation of SK channels facilitates the synaptically mediated burst induction and in some cases, induces burst firing in vitro (Shepard and Bunney, 1991). SK channels form a signalling complex with calmodulin as a calcium detector and channel opening depends solely on submembrane changes of the intracellular Ca concentration. In SN neurons, SK channels are activated almost exclusively via T-type Ca channels. The inhibition of T-type channels alone switched the firing pattern of some DA neurons to an intrinsic burst-firing mode; blocking of both SK and T-type channels increases the burst occurrence significantly (Wolfart and Roeper, 2002). Reduction of small conductance calcium-activated potassium current (SK) by application of apamin potentiates the excitatory effect of ethanol on VTA DAergic neurons (Brodie et al., 1999). The injection of depolarizing current revealed that in vitro the DAergic neurons could not maintain firing at high frequencies and displayed stronger frequency adaptation in comparison with GABAergic neurons. Accommodation continued throughout higher current injections; in addition, depolarization block could be observed upon strong depolarization (Richards et al., 1997).
Most *in vitro* electrophysiological studies have considered DA midbrain neurons mainly as a single population. However, *in vivo* studies have highlighted functional differences between subgroups of DA neurons (Chiodo et al., 1984; Shepard and German, 1988).

1.2. Burst firing in dopaminergic neurons.

In vivo a second activity pattern – burst firing, in which DA neurons fire spikes in groups of 3 to 8 action potentials of decreasing amplitude and increasing duration, can be observed. It shows little dependency on the baseline firing rate, although increases in activity typically cause a transition into the burst firing mode (Grace, 1988). Burst firing is associated with the unexpected appearance of rewards or stimuli predicting reward (Schultz, 1998). Thus, determining the sources of afferent input that are responsible for the generation of burst firing is crucial in understanding the function of ascending DA systems. Burst firing in DA neurons is dependent, at least in part, on glutamate input, because blockade of glutamate receptors suppresses this activity pattern in these cells (Charlety et al., 1991). One of the principal glutamate inputs to the ventral tegmental area (VTA) arises from the PFC (Sesack and Pickel, 1992). Moreover, PFC stimulation increases burst firing of DA neurons (Gariano and Groves, 1988), whereas inactivation of the PFC produces the opposite effect (Svensson and Tung,

1989). The inputs from the PPTN and subthalamic nucleus also produce burst firing in VTA (Overton and Clark, 1997). In vitro, a burst-like pattern, somewhat different from "natural" bursts, can be elicited by application of nickel, alone or in combination with apamin, which blocks a slow afterhyperpolarization (Wolfart and Roeper, 2002), or by NMDA together with apamin (Seutin et al., 1993).

1.3. Dopamine release

Impulses of dopamine neurons at intervals of 20–100 ms lead to a much higher dopamine concentration in striatum than the same number of impulses at intervals of 200 ms (Garris and Wightman, 1994). This nonlinearity is mainly due to the rapid saturation of the dopamine reuptake transporter, which clears the released dopamine from the extrasynaptic region. The same effect is observed in nucleus accumbens (Wightman and Zimmerman, 1990) and occurs even with longer impulse intervals because of sparser reuptake sites. Dopamine release after an impulse burst of 300ms is too short for activating the autoreceptor-mediated reduction of release or the even slower enzymatic degradation. Thus a bursting dopamine response is particularly efficient for releasing dopamine. Single impulse releases ~1,000 dopamine molecules at synapses in striatum and nucleus accumbens. This leads to immediate synaptic dopamine concentrations of 0.5–3.0 mM (Garris et al. 1994; Kawagoe et al. 1992). At 40 ms after release onset, ~90% of dopamine has left the synapse, some of the rest being later eliminated by synaptic reuptake. At 3–9 ms after release onset, dopamine concentrations reach a peak of ~250 nM when all neighboring varicosities simultaneously release dopamine. Concentrations are homogeneous within a sphere of 4 μm diameter (Gonon 1997), which is the average distance between varicosities (Doucet et al. 1986; Groves et al. 1995). Maximal diffusion is restricted to 12 μm by the reuptake transporter and is reached in 75 ms after release onset. Concentrations would be slightly lower and less homogeneous in regions with fewer varicosities or when 100% of dopamine neurons are activated, but they are two to three times higher with impulse bursts. Thus the reward-induced, mildly synchronous, bursting activations in 75% of dopamine neurons may lead to rather homogeneous concentration peaks in the order of 150–400 nM. Total increases of extracellular dopamine last 200 ms after a single impulse and 500–600 ms after multiple impulses of 20–100 ms intervals applied during 100–200 ms (Chergui et al., 1994). The extrasynaptic reuptake transporter subsequently brings dopamine concentrations back to their baseline of 5–10 nM. Thus in contrast to classic, strictly synaptic neurotransmission, synaptically released dopamine diffuses rapidly and reaches short peaks of regionally homogenous extracellular concentrations (Schultz, 2002).

1.4. Subgroups of dopaminergic neurons: electrophysiological and functional differences.

About a half of dopaminergic neurons in SN and VTA express the calcium-binding protein calbindin (CB). A number of differences are found between CB-negative and CB-positive neurons. In the substantia nigra pars compacta (SNc), a dorsal and a ventral tier of DA neurons have been described that project to neurochemically different compartments in the striatum (Maurin et al., 1999). In addition, some DA neurons are found in substantia nigra pars reticulata (SNr). Ventral tier SNc and SNr DA neurons that do not express calbindin D_{28}-k (CB−), project to striatal patch compartments and in turn receive innervation from striatal projection neurons in the matrix. Conversely, calbindin-positive (CB+) dorsal tier SNc DA neurons project to the striatal matrix while receiving input from the limbic patch compartment. CB+ and CB− DA neurons have also been described in the VTA but little is known about their axonal targets (Barrot et al., 2000). The exact function of calbindin in DA neurons is unknown, but CB+ DA neurons appear to be less vulnerable to degeneration in Parkinson's disease and its animal models (Liang et al., 1996)

The calbindin-positive and calbindin-negative subpopulations of DA neurons in SN and VTA neurochemically and anatomically identified DA subpopulations possess significant electrophysiological differences in particular in response to hyperpolarizing current injections and in pacemaker frequency control (Neuhoff et al., 2002). In contrast, within individual neurochemically defined DA subpopulations, variations of these functional properties are not strongly correlated to their mediolateral or ventrodorsal positions within the respective nucleus. The anatomical distributions of these functionally and neurochemically distinct DA subpopulations correlate with the anatomical topography of DA midbrain systems (Maurin et al., 1999, Joel and Weiner, 2000). This might suggest that DA populations with distinct axonal targets, like CB+ and CB- SN neurons, possess also different postsynaptic properties. In the VTA, the distribution of CB+ DA neurons that display the most distinct phenotype with irregular discharge at higher frequencies combined with a prolonged postinhibitory hypoexcitability best match the localization of mesoprefrontal DA neurons (Chiodo et al., 1984). In contrast, the larger, calbindin-negative (VTA/CB-) DA neurons are more likely to constitute the mesolimbic projections (Oades and Halliday, 1987). However, the direct functional analysis of retrogradely labelled DA midbrain neurons is not yet made. It was also found that the differences in I_h currents contribute to selective pacemaker control and subthreshold properties in identified DA subpopulations. Differences in I_h charge densities are

an important mechanism responsible for the functional diversity of DA neurons. Under the assumption of similar unitary I_h channel properties, these different I_h charge densities would correspond to different densities of functional I_h channels. Qualitative single-cell RT-mPCR experiments have shown that DA SN neurons coexpress three of the four I_h channel subunits, HCN2, HCN3, and HCN4 (Franz et al., 2000). However, the molecular composition of native neuronal I_h channels that might exist as homomeric or heteromeric complexes as well as the possible differential I_h channel subunit expression between different DA subpopulations remains unclear. In this context, quantitative differences in HCN subunit expression might also play a significant role. Relevant functional differences in subthreshold behavior remain even during complete inhibition of I_h channels between the different DA subpopulations. This indicates that other ion channels, such as SK3 channels (Wolfart et al., 2001) are also differentially expressed in distinct DA populations. The described irregular firing DA VTA neurons with low SK3 channel density are likely to correspond to the calbindin-positive VTA subpopulation. In addition, it was shown by quantitative single-cell real-time PCR that differences in transcript numbers for Kv4α and Kv4β subunits control the A-type potassium channel density and pacemaker frequency in DA SN neurons (Liss et al., 2001). Other obvious candidates that might contribute to functional diversity are persistent sodium channels (Grace, 1991) and low-threshold calcium channels (Cardozo and Bean, 1995). Only in SN/CB− neurons I_h channels are directly involved in pacemaker frequency control. Similar results have been obtained by extracellular recordings in DA neurons (Seutin et al., 2001). Selective pacemaker control by I_h channels has two important consequences. First, because I_h channels significantly contribute to the resonance profile of neurons (Hutcheon and Yarom, 2000), the active I_h channel pool will selectively increase the stability of regular, tonic discharge in SN/CB− DA neurons. I_h channels are likely to do this in concert with the high density of calcium-activated SK3 channels that are also present in these SN neurons and control frequency and stability of the pacemaker (Wolfart et al., 2001). *In vivo* studies have shown that this DA subtype discharges more regularly and less often in burst mode compared with VTA DA neurons (Grace and Bunney, 1984). In this context, it is important that the transition between single spike and burst mode (i.e., tonic and phasic DA signaling) is regarded as an essential element in the signal processing of the DA system (Schultz, 1998). In addition to pacemaker control, the differences in I_h channel density could also lead to distinct modes of phasic postsynaptic integration. Whereas SN/CB− DA neurons show an I_h channel-dependent transient, postinhibitory excitation, VTA/CB+ DA neurons display a pronounced postinhibitory inhibition. These results indicate that the differences in I_h channel density in

DA neurons might be important for the integration of GABAergic signaling, which represents the most abundant (>70%) synaptic input to DA neurons(Grace and Bunney, 1985). These postsynaptic differences are well suited to amplify the different pattern of GABA-mediated indirect rebound excitation or direct inhibition that have both been observed in DA neurons *in vivo* (Kiyatkin and Rebec, 1998). It has been postulated that SN/CB− DA neurons operate in a closed striato-nigro-striatal loop providing phasic DA release induced by concerted and precisely timed disinhibition from nigral and pallidal GABAergic input, whereas SN/CB+ DA neurons as well as VTA DA neurons are directly inhibited by striatal input in an open-loop configuration with less temporal precision (Maurin et al., 1999). Thus, the differences in I_h channel density could contribute to the different polarity and temporal structure of GABAergic integration in DA neurons.

1.5. Differential vulnerabilities to neurodegeneration of DA midbrain neurons are associated with distinct functional phenotypes

Anatomical position and differential expression of calbindin were shown to be associated with differential vulnerability of DA neurons to neurodegeneration in Parkinson's disease and its related animal models (Liang et al., 1996). There is consensus that the calbindin-negative SN neurons are significantly more vulnerable compared with the calbindin-positive SN/CB+ and VTA neurons. However, studies on the calbindin-KO mouse have shown that this protein is not causally involved in conferring resistance to neurotoxins and thus might only be used as a marker for less vulnerable cells in the SN (Airaksinen et al., 1997). In this context, it is noteworthy that only the highly vulnerable class of DA neurons possesses the strong rebound activation, which might render these neurons more susceptible to glutamatergic input (Beal, 2000). In addition, the most vulnerable DA neurons possess the highest density of I_h channels. Mitochondrial dysfunction, which is regarded as an important trigger factor of Parkinson's disease (Beal, 2000), might lead to tonic activation of ATP-sensitive potassium (K-ATP) channels and consequently to chronic membrane hyperpolarization (Liss et al., 1999a). Indeed, this tonic activation of K-ATP channels has been demonstrated in DA neurons in the *weaver* mouse, a genetic model of dopaminergic neurodegeneration (Liss et al., 1999b). However, K-ATP channel-mediated membrane hyperpolarization will activate I_h channels and thus counteract hyperpolarization and also lead to sodium loading. Thus, differential characteristics of calbindin-positive and calbindin-negative DA neurons might result in different pathophysiological responses to metabolic stress and contribute to the differential vulnerability of DA neurons to neurodegeneration (Neuhoff et al., 2002).

2. Electrophysiology and morphology of GABAergic cells in VTA and SN.

Most if not all TH-negative neurons in SN and VTA are GABAergic, called "secondary" in some papers. They are immunopositive for glutamate decarboxylase (GAD). The GABA neurons in the SN pars reticulata possess a number of well defined features (Richards et al., 1997): they have short duration action potentials (0.45 ± 0.03 ms halfwidth), no apparent rectifying currents, no low threshold calcium spikes, are spontaneously active (7.4 ± 3.7 Hz), display little frequency adaptation and could maintain high firing rates. Morphological reconstruction of neurobiotin-filled neurons revealed that the pars reticulata GABA neurons have more extensive local dendritic arborization than the dopamine neurons from either the pars reticulata or the pars compacta. The electrophysiology of the GABA neurons suggests that input activity is translated linearly to spike frequency. These GABA neurons probably represent the projection neurons of the pars reticulata, and it is thus likely that this basal ganglia output is frequency coded (Richards et al., 1997). In SN zona compacta GABAergic neurons (5% of the total) had properties similar to GABAergic neurons in SN pars reticulata. These neurons fired spontaneous action potentials at frequencies greater than 10 Hz, or were quiescent (Lacey et al., 1989); had action potentials less than 1 msec in duration and did not show time-dependent inward rectification with step hyperpolarization. GABAergic neurons were not affected by dopamine but were hyperpolarized by baclofen, GABA, and the mu opioid receptor agonist Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) (Lacey et al., 1989).

In the VTA, neurons without detectable TH immunoreactivity lie in close proximity to TH-labelled cells and are presumed to be GABAergic neurons (Nagai et al., 1983). These neurons appear to represent a heterogeneous population whose neurochemical identity, projections, innervation, and physiological significance are less clear than that of DA neurons in the VTA. VTA non-DA neurons recorded extracellularly in halothane-anesthetized rats were distinguished from VTA DA neurons by location, spontaneous activity, axonal conduction velocity, refractory period, and orthodromic-driven activity (Steffensen et al., 1998). The most distinguishing feature of VTA non-DA neurons was their fast spontaneous activity (19.1 ± 1.4 Hz) relative to DA neurons and their uninterrupted phasic activity characterized by alternating 0.5-2 sec "on" and 0.5-2.0 sec "off" periods (mean period, 0.43 ± 0.07 Hz). No bursting activity was observed in any of the VTA non-DA neurons studied. Unfiltered recordings of VTA non-DA neuron spikes revealed biphasic action potentials, characterized by a prominent, initial negative-going component followed by a small positive-going component. The mean duration of the negative-going spike measured at half-maximal amplitude was 310 ± 10 μsec. VTA non-DA neurons were found in clusters of neurons whose

spontaneous activity appeared to be homogeneous. VTA non-DA neurons had a mean resting membrane potential of −61.9 ± 1.8 mV, and their mean spike amplitude was 68.3 ± 2.1 mV. The "on" period of spontaneous VTA non-DA activity was accompanied by a 9.4 ± 0.9 mV depolarization. Spontaneous and orthodromic IC-evoked VTA non-DA intracellular spikes were preceded by an EPSP whose mean amplitude was 7.6 ± 0.3 mV. There appeared to be little or no spontaneous EPSP activity during the "off" phase. They are rapidly firing, nonbursting neurons with reciprocal innervation from the cortex and inhibitory input from the NAcc indicating that these neurons influence and are influenced by cortical and limbic structures. They also contain GABA immunoreactivity and receive excitatory synapses from unlabelled terminals and symmetric inhibitory synapses from terminals that sometimes contain GABA immunoreactivity. These findings indicate that VTA non-DA neurons are GABAergic and are also subject to GABA inhibition. The prevailing view was that VTA non-DA neurons are local circuit interneurons (Beart and McDonald, 1980); however, VTA non-DA neurons also project to the cortex and ventral striatum (Thierry et al., 1980). Furthermore, the neurotransmitter used by both local and projection neurons in the VTA, similar to that used by non-DA neurons in the substantia nigra, is thought to be GABA (Nagai et al., 1983). This is supported by studies showing that GABAergic terminals provide synaptic input to DA neurons in the VTA (Bayer and Pickel, 1991). VTA non-DA neurons were driven antidromically by IC stimulation, indicating that they were not only local circuit interneurons but that they project to cortical sites as well. Many studies have emphasized the role of intrinsic membrane properties to explain the rate and pattern of firing of midbrain DA neurons (Grace and Bunney, 1984; Lacey et al., 1989b; Kang and Kitai, 1993). There are, however, significant differences between the spontaneous activity of SN and VTA DA neurons *in vitro* and *in vivo* (Wilson et al., 1977; Kita et al., 1986), suggesting that afferent input plays a role in modulating the activity, particularly the firing pattern, of these neurons. The VTA non-DA neurons described in the *in vitro* study share similar characteristics to those recorded in vivo, including an action potential duration of <1.0 msec, a mean resting membrane potential of −60.8 ± 2.6 mV, and a lack of rectification to hyperpolarizing current steps. When considered together, these findings strongly suggest that the firing rate of VTA non-DA neurons is a function of afferent input.

3. Coexpression of TH and GAD in a subgroup of midbrain neurons

By using immunocytochemistry, it has been reported that a subpopulation of nigrotectal neurons coexpresses TH and GAD (glutamic acid decarboxylase) (Campbell et al., 1991). These findings suggested the possibility that, besides the nondopaminergic GABAergic nigrostriatal cells, a subgroup of dopaminergic nigrostriatal neurons contains GABA. The electrophysiological properties and functions of this group are still unknown. These findings suggested the possibility that, besides the nondopaminergic GABAergic nigrostriatal cells, a subgroup of dopaminergic nigrostriatal neurons contains the neurotransmitter GABA. The confirmation of this hypothesis would indicate that the relative role of GABAergic transmission in the nigrostriatal pathway is more important than currently accepted. Studies carried out during the last two decades have shown the existence of two GAD isoforms, each encoded by a different gene, and differing in molecular size and intraneuronal distribution (Denner and Wu, 1985; Kaufman et al., 1991). One of them, with 67 000 Dalton (GAD67), is widely distributed throughout the neuron, and the other, with 65 000 Dalton (GAD65), is localized mostly in axon terminals. Although immunocytochemistry is a useful technique for identifying GABAergic neurons, in some structures such as the SN, the low concentration of GAD in somata, together with its high concentration in striatonigral terminals, makes it difficult to consistently visualize GAD-containing neurons, particularly those containing GAD65. The possibility that nigrostriatal dopaminergic neurons express GAD67 and/or GAD65 or their messenger, was studied by combining immunocytochemistry and in situ hybridization for both GAD isoforms with immunocytochemistry for TH and retrograde neuronal tracers (Gonzalez-Hernandez et al., 2001). They found GAD67 immunoreactivity in both the neuropil and somata of the SN. The neurons expressing GAD67 were localized mainly in the SN pars reticulata, and a few in the SN pars compacta, particularly in its medial region. Sparse GAD67-immunoreactive neurons were also found in neighbouring DA midbrain centres, the retrorubral field (A8) and VTA (A10). However, in the immunofluorescence material, GAD67 and TH double-labelled neurons were not detected. In contrast to GAD67, GAD65 immunoreactivity was only found in nigral neuropil, with a higher intensity in the dorsomedial region of the SNc. In the SN pars reticulata the distribution pattern of neurons containing GAD67 mRNA is similar to that of neurons containing GAD65 mRNA (Esclapez et al., 1994), suggesting that many of them contain mRNA for both GAD isoforms. However, in the SN pars compacta, particularly in its rostromedial region, the number of GAD65 mRNA-positive neurons was higher than that of GAD67mRNA-positive neurons, suggesting that in this region a subpopulation of nigral cells

contains GAD65 mRNA but not GAD67mRNA. The combination of in situ hybridization for GAD mRNAs and immunocytochemistry or immunofluorescence for TH demonstrated that a number of them express TH. Interestingly, practically all neurons showing double labelling were GAD65 mRNA+TH positive, corresponding to 9.8% of the total number of TH immunoreactive cells, whereas only one was GAD67mRNA+TH positive. With respect to their topographic distribution, most GAD65 mRNA+TH cells (57.6%) were localized in the medial third of the SNC, where 19.5% of TH cells contained GAD65 mRNA. A significant number of double labelled neurons (24.6%) were also found in the VTA. Retrograde (fluorogold) striatal labeling combined with GAD65 mRNA+TH double labelling in order to investigate the possibility that these neurons might project to the striatum revealed that 9.4% of nigrostriatal neurons studied contained both TH and GAD65 mRNA. Thus, a third pathway formed by approximately 10% of dopaminergic nigrostriatal neurons that contain GAD65 mRNA but not GAD67mRNA and GAD67, and are preferentially localized in the SN pars compacta and VTA. These distinctive features support the existence of two different GABAergic nigrostriatal pathways. Although most nigral GABAergic neurons, perhaps also those GABAergic/nondopaminergic cells projecting to striatum, contain both GAD mRNAs (Esclapez et al., 1994), dopaminergic/GABAergic cells only express GAD65 mRNA. This fact may be of interest in the GABAergic transmission in the nigrostriatal system as both forms, besides differing in their molecular sizes, also differ in other biochemical properties. GAD67 is widely distributed throughout the neuron as an active holoenzyme form (holoGAD), practically saturated with cofactor (Kaufman et al., 1991). It has been associated with functions requiring relatively high levels of GAD synthesis, such as the provision of constitutive levels of GABA transmitter (Esclapez et al., 1994). GAD65, in contrast, is present in axon terminals as an inactive apoenzyme (apoGAD, without bound cofactor), providing a reservoir of GAD (Kaufman et al., 1991). The conversion of GAD65 into its active form, by binding pyridoxal phosphate, is regulated by energy metabolites, with inorganic phosphate increasing, and ATP decreasing, the association rates between pyridoxal phosphate and GAD. In this context, these data suggest different functions for the two GABAergic nigrostriatal projections. The projection arising from the SN pars reticulata that expresses GAD67 would be channelled towards maintaining the basal levels of GABA for tonic neuronal activity and long-lasting demands of GABA synthesis, whereas the projection arising from the SN pars compacta and the VTA that expresses GAD65 would synthesize GABA in response to local demands, playing an essential role in the short term regulation of the GABA transmitter pool.

It is known that GABAergic medium spiny cells projecting to the SN pars reticulata and globus pallidus are the main target of the dopaminergic component of the mesostriatal projection (Smith and Bolam, 1990). The fact that dopaminergic terminals contain GABA receptors (Ronken et al., 1993), and that dopaminergic/GABA terminals contain GAD65, which is quickly activated by energy metabolites, suggest that GABA released from dopaminergic/GABAergic cells can exert a short auto-regulatory mechanism on the mesostriatal system. In these dopaminergic cells, GABA cotransmission could play a modulatory role, protecting these neurons from excessive activity, and thus, exerting a neuroprotective effect (Gonzalez-Hernandez et al., 2001). This hypothetical autoregulatory mechanism could help to explain why in Parkinson's disease dopaminergic neurons exhibiting a higher resistance to degeneration are localized in the medial portion of the SN pars compacta and the VTA, coinciding with those expressing GAD65 mRNA described in Gonzalez-Hernandez et al. study.

By contrast, bearing in mind the topographical organization of the mesostriatal projections in the mediolateral axis (Beckstead et al., 1979), the fact that dopaminergic/GABAergic cells are restricted to the medial region of the SN pars compacta and the VTA suggests that they project to the medial striatum and nucleus accumbens. Because the mesoaccumbens projection has been related extensively to reward phenomena (Wise, 2002), and medial striatal regions receive projections from the prefrontal and associative cortex, dopaminergic/GABAergic mesostriatal cells should act on rewarding states on prefrontal and associative cortex-related functions rather than on those related to the sensorimotor cortex, which projects to lateral striatal regions (Deniau et al., 1996)

4. Efferents and afferents of SN and VTA neurons.

Neurons of SN pars reticulata together with the internal segment of the globus pallidus form the major outputs of the basal ganglia. Neurons of SN pars reticulata send axons to the ventrolateral (VL) and ventromedial (VM) nuclei of the thalamus, the superior colliculus (SC) and medial pontine reticular formation (PRF). 42,6% of SNr cells inhibit the PRF, about a half of them had branching axons to SC and/or thalamus (Niijima and Yoshida, 1982). Nishimura Y. et al (1997) examined the patterns of distribution and collateral projections of the two major groups of nigrothalamic neurons, i.e., nigro-MD and nigro-VM neurons. A clear tendency was observed that nigro-MD neurons were distributed more ventrally than nigro-VM neurons. The nigro-MD neurons were found to send axon collaterals to the superior

colliculus more frequently than the nigro-VM neurons. Additional projection fibers from the SN pars reticulata terminate in several other thalamic nuclei, including the ventrolateral, centromedial, centrolateral, paracentral and parafascial, however, these nigrothalamic projections are not as dense as those to MD and VM. It is well known that the nigrotectal pathway, through which projection fibers from the SNr reach the deep layers of the SC, plays a crucial role in the onset of saccadic eye movements. The deep layers of the SC, a target for axon collaterals of nigro-MD neurons, receive input from the frontal eye field and send output to the paralaminar zone of MD. The VM sends projection fibers to layer I of the frontal cortex and nigrotalamic and cerebellothalamic inputs converge, to some extent, into the VM. Additionally, a small number of nigrothalamic neurons were found to send axon collaterals to the pontine reticular formation. Another main target region for nigroreticular projections is the pedunculopontine tegmental nucleus (PPTN). SN pars reticulata neurons, giving rise to nigroreticular fibers to the PPTN, often send axon collaterals to the thalamus (Nishimura et al., 1997).

SN pars compacta dopamine neurons project mainly in dorsal striatum (caudate nucleus and putamen), forming the mesostriatal dopaminergic system. The spiny projection neurons of the neostriatum are a site at which dopamine inputs from the substantia nigra converge with excitatory inputs from the cerebral cortex. Both dendrites of spiny neurons, and the cell bodies of cholinergic interneurons, receive dopaminergic input from SN (Freund et al., 1985). The organization of these two populations with respect to the striatal projection fields suggests that the substantia nigra might control the flow of cortical information through the striatum via two different modalities, based respectively on a closed nigrostriatal loop involving the proximal neurons, and an open loop involving the distal ones (Maurin et al., 1999).

A10 dopamine neurons project from the VTA to the nucleus accumbens (NAcc), amygdala, hippocampus and prefrontal cortex, forming the mesocorticolimbic dopamine system (Albanese and Minciacchi, 1983). VTA GABAergic neurons also project to the prefrontal cortex, (NAcc) and regulate the activity of VTA dopamine neurons via local axon collaterals (Steffensen et al., 1998; van Bockstaele and Pickel, 1995).

4.1. Striatum.

The ventral striatum (nucleus accumbens) plays a major role in mediating motivation and reward. Studies of this striatal region have focused on its role in influencing motor outcome by funnelling information from the limbic system to the motor system (the "limbic/motor interface")(Mogenson et al., 1980). Nauta et al. (Nauta et al., 1978) first proposed that dopamine plays a role in this limbic/motor interaction through the accumbens projection to the substantia nigra, which in turn projects to the dorsal striatum. However, the dorsal striatum is involved in more than motor function. In primates it is linked not only to motor and premotor cortical areas but to the whole frontal cortex, including the dorsolateral prefrontal cortex (Haber et al., 2000). The outflow of the striatum reaches major extrapyramidal motor centers such as globus pallidus, substantia nigra, and subthalamic nucleus, and the input to this structure, arising from the neocortex, limbic system, and midbrain, suggests that it plays a complex integrative role in adaptive motor actions (Kelley et al., 1997).

The nucleus accumbens is best known for its role in mediating the reinforcing and rewarding properties of drugs of abuse. Drugs such as cocaine, heroin, alcohol, and even nicotine are hypothesized to produce their rewarding effects via activation of accumbens dopamine (Wise, 2002), and it has been recently postulated that chronic neuroadaptations in this system may underlie the addiction process. Parallel research has indicated, not surprisingly, that the nucleus accumbens and its associated circuitry subserve behaviors linked to natural or biological rewards, such as feeding, drinking, sex, exploration, and appetitive learning (Robbins and Everitt, 1996). Neurons in the monkey ventral striatum are sensitive to both primary and conditioned rewards (Schultz et al., 1993). Moreover, neuronal response plasticity has been demonstrated in striatal neurons during behavioral learning. During acquisition of sensorimotor conditioning in monkeys, in which a cue predicts delivery of juice reward, there is a progressive increase in the number of tonically active neurons that respond to the cue (Aosaki et al., 1994). Indeed, an important theory of striatal function posits that this structure is crucial for the acquisition and performance of relatively automatic learned "habits," or basic stimulus-response learning (Packard and White, 1990). Lesions of the ventral or dorsal striatum have been found to impair acquisition on a variety of learning tasks, particularly when animals are required to use fixed cues to improve performance. A basic rule of positive reinforcement is that motor responses will increase in magnitude and vigour if followed by a rewarding event. It is likely, therefore, that the nucleus accumbens may serve as a substrate for reinforcement learning. In vivo electrophysiological recording experiments suggest that ventral and dorsal striatal neurons are sensitive to motivationally significant stimuli in the environment and show firing properties during appetitive conditioning tasks

consistent with adaptive changes during learning (Schultz et al., 1993). The enhanced dopaminergic signal within the accumbens, provided by both food deprivation and availability of food reward (Wilson et al., 1995), undoubtedly plays an important role in modulating response learning.

Several recent studies have implicated the shell subregion of accumbens (AcbSh) as an important component of a neural system specifically involved in the mediation of feeding behavior. Inhibition of neurons in the AcbSh by administration of excitatory amino acid antagonists (Stratford et al., 1998) or GABA agonists (Stratford and Kelley, 1997) elicits intense feeding in satiated rats. These treatments appear to affect feeding behaviour specifically, because they do not increase water intake, noningestive gnawing, or locomotor activity (Stratford et al., 1998). As such, the effect does not appear to be the result of a general behavioural activation. The majority of cells projecting from the AcbSh are medium spiny neurons that use GABA as a neurotransmitter, and both symmetric inhibitory GABAergic terminals and neurochemically uncharacterized asymmetric (presumably excitatory) terminals have been shown in apposition to the axon hillock of these neurons (Meredith et al., 1993), placing them in a position to exert a powerful influence on the output of these cells. Thus, the inhibition of GABAergic AcbSh projection neurons through actions at glutamate and GABA receptors located on those cells, then disrupting GABA transmission in the terminal fields of those neurons, may also elicit feeding. Currently, the locations of the relevant terminal fields are unknown; however, a likely candidate appears to be the lateral hypothalamus (LH). In the paper by Maldonado-Irizarry et al that initially described the elicitation of feeding from the AcbSh, it is noted that the intensity of the feeding is similar to the LH stimulation-induced feeding. They subsequently demonstrated a functional relationship between these two brain regions by showing that AcbSh-mediated feeding could be attenuated by injections of the $GABA_A$ receptor agonist muscimol into the LH (Maldonado-Irizarry et al., 1995). Furthermore, it is known that neurons in the AcbSh project directly to the LH (Kirouac and Ganguly, 1995) and that electrical or chemical stimulation of LH neurons can induce robust feeding in satiated animals. Bilateral microinjections of muscimol into the AcbSh elicited intense feeding in satiated rats and greatly increased consumption of chow over a 2 hr period compared with intake after vehicle injections (Stratford and Kelley, 1999). Injections of muscimol into the AcbSh also greatly increased the number of cells showing Fos-like immunoreactivity (Fos-LI) in the LH. Although a significant increase in Fos-LI was observed throughout the rostrocaudal extent of the LH, the largest increase was seen in the perifornical region of the nucleus. Cell counts on comparable LH sections demonstrated that AcbSh

injections of muscimol significantly increased the number of neurons exhibiting Fos-LI compared with saline-injected rats. Large increases in Fos-LI also were observed in a number of brain regions other than the LH, including the lateral septum (LS) and dorsolateral preoptic region, the PVN, and the caudal LH- ventral tegmental area (VTA) transition zone in the vicinity of the supramamillary nucleus (SuM), the VTA and medial substantia nigra pars compacta. Simultaneous administration of the $GABA_A$ receptor agonist muscimol into the AcbSh and saline into the perifornical LH elicited intense feeding in satiated rats. This feeding response was dose-dependently attenuated by injections of the selective NMDA antagonist AP-5 into the perifornical LH. Bilateral injections of the selective $GABA_A$ receptor blocker bicuculline or the selective $GABA_B$ receptor blocker saclofen did not alter food or water intake significantly in satiated rats (Stratford and Kelley, 1999). In summary, the feeding elicited by injecting muscimol into the AcbSh is accompanied by an increase in the synthesis of Fos in neurons located in the LH, suggesting that these cells are increasing their firing rates in response to the stimulus. Furthermore, the NMDA receptor-mediated activation of LH neurons is necessary for the expression of the AcbSh-mediated feeding. Neurons in the AcbSh, however, do not appear to control the firing rate of LH neurons through a direct GABAergic projection to the LH. Blocking GABA receptors in the medial ventral pallidum, a brain region anatomically interposed between the AcbSh and LH, induces robust feeding in rats (Stratford et al., 1999). Thus, these findings raise the interesting possibility that an AcbSh-VPm-LH circuit is involved in the control of food intake.

The GABAergic neurons in the striosomes (patches) of the striatum project in a broadly topographic and partly overlapping manner to dopamine neurons in nearly the entire pars compacta of substantia nigra, whereas neurons of the much larger striatal matrix contact predominantly the non-dopamine neurons of pars reticulata of substantia nigra, besides their projection to globus pallidus (Smith and Bolam, 1990). Neurons in the ventral striatum project in a non-topographic manner to both pars compacta and pars reticulata of the medial substantia nigra and to the ventral tegmental area (Schultz, 1998). The GABAergic striatonigral projection may exert two distinctively different influences on dopamine neurons, a direct inhibition and an indirect activation (Grace and Bunney, 1985). The latter is mediated by striatal inhibition of pars reticulata neurons and subsequent GABAergic inhibition from local axon collaterals of pars reticulata output neurons onto dopamine neurons. This constitutes a double inhibitory link and results in net activation of dopamine neurons by the striatum. Thus striosomes and ventral striatum may monosynaptically inhibit and the matrix may indirectly activate dopamine neurons (Schultz, 1998).

4.2. Prefrontal cortex.

One of the principal glutamate inputs to the ventral tegmental area (VTA) arises from the prefrontal cortex (PFC) (Sesack and Pickel, 1992). Moreover, PFC stimulation increases burst firing of DA neurons (Tong et al., 1996), whereas inactivation of the PFC produces the opposite effect. These effects may be mediated by the known monosynaptic projection from the PFC to DA neurons within the VTA. PFC afferents may target DA neurons that project to the NAc or those that project back to the PFC, because there is substantial overlap between the distribution of PFC terminals and the soma and dendrites of both mesoaccumbens and mesoprefrontal neurons within the VTA (Sesack and Pickel, 1992). In addition, PFC stimulation produces excitatory responses in mesocortical or mesoaccumbens neurons that exhibit the physiological characteristics of DA cells(Gariano and Groves, 1988). Neurochemical studies also indicate that PFC afferents target the DA cell populations that project to the NAc or to the PFC. Stimulation of the PFC increases levels of extracellular DA within the NAc, an effect that is blocked by infusion of glutamate antagonists into the VTA but not into the NAc (Taber and Fibiger, 1995). Inactivation of the PFC produces the opposite response, indicating a role of the PFC in the regulation of tonic levels of NAc DA. Stimulation of the PFC by local infusion of glutamate agonists also increases DA levels within the PFC, whereas glutamate antagonist infusion has the opposite effect. These effects may be attributable to changes in the activity of PFC neurons that project to mesoprefrontal DA cells, although mechanisms that are local to the cortex cannot be excluded. Finally, in addition to the extensively studied DA projections of the VTA, recent studies have also demonstrated that GABA-containing neurons project from the VTA to both the NAc (van Bockstaele and Pickel, 1995) and to the PFC (Carr and Sesack, 2000). It is not known if these GABA-containing projection systems receive synaptic input from the PFC. However, both anatomical (Sesack and Pickel, 1992) and electrophysiological studies have demonstrated monosynaptic contacts of PFC afferents onto non-DA neurons in the VTA. Thus, both GABA mesoaccumbens and mesocortical neurons may receive PFC synaptic input. Previous investigations have demonstrated that PFC terminals synapse on the dendrites of DA and non-DA neurons in the VTA. To address whether PFC afferents innervate different populations of VTA neurons that project to the nucleus accumbens (NAc) or to the PFC, a triple labeling method was used that combined peroxidase markers for anterograde and retrograde tract-tracing with pre-embedding immunogold-silver labeling for either tyrosine hydroxylase (TH) or GABA (Carr and Sesack, 2000). Within the VTA, PFC terminals formed asymmetric synapses onto dendritic shafts that were immunoreactive for either TH or GABA. PFC terminals also synapsed on VTA dendrites

that were retrogradely labelled from the NAc or the PFC. Dendrites retrogradely labelled from the NAc and postsynaptic to PFC afferents were sometimes immunoreactive for GABA but were never TH-labeled. Conversely, dendrites retrogradely labeled from the PFC and postsynaptic to PFC afferents were sometimes immunoreactive for TH but were never GABA-labeled. These results provide the first demonstration of PFC afferents synapsing on identified cell populations in the VTA and indicate a considerable degree of specificity in the targets of the PFC projection. The unexpected finding of selective PFC synaptic input to GABA-containing mesoaccumbens neurons and DA-containing mesocortical neurons suggests novel mechanisms through which the PFC can influence the activity of ascending DA and GABA projections(Carr and Sesack, 2000).

It has been proposed that VTA DA neurons may be regulated by both a direct excitatory cortical input to DA neurons and, indirectly, an inhibitory input comprising cortical excitatory inputs onto VTA non-DA neurons (Wang and French, 1995). Therefore, the excitability of VTA DA neurons would result from the net effect of direct excitation and indirect inhibition from non-DA neurons by cortical afferents. The latter may explain why the NMDA receptor blockers MK-801 and phencyclidine excite DA neurons in vivo, increase DA release in the NAcc, and produce hyperlocomotion (Steffensen et al., 1998). Corticofugal glutamatergic projections to VTA DA neurons as well as glutamate receptors have also been implicated in the development of behavioral sensitization to psychostimulants, an animal model for the intensification of drug craving believed to underlie human drug addiction. Sensitization results, in part, from a long-term change in mesocorticolimbic DA transmission and may involve a disinhibition of dopamine neurons (Steketee and Kalivas, 1991). The disinhibition of DA neurons may result from decreased excitatory corticofugal drive to VTA non-DA neurons or from increased GABAergic drive from the NAcc onto VTA non-DA neurons. Because of their wideband firing activity, dependency on NMDA receptor-mediated cortical input, and inhibitory modulation by the NAcc, VTA non-DA neurons may contribute to plasticity in the complex neuronal circuits underlying behavioral sensitization. Steffensen et al (Steffensen et al., 1998) hypothesize that these neurons receive a physiologically relevant NMDA receptor-mediated input that paces GABA inhibition to DA neurons in a manner similar to the role played by thalamic inputs to substantia nigra pars reticulata GABAergic neurons in mediating inhibition of SNc DA neurons (Tepper et al., 1995)

5. Physiological functions and consequences of DAergic and GABAergic neuronal activity

Ventral tegmental area (VTA) dopaminergic and GABAergic neurons are critically involved in brain mechanisms of reward, reinforcement and emotional arousal (Wise and Rompre, 1989). The firing of dopamine neurons in this region is closely correlated with the availability of primary rewards (food, water, sex) (Schultz, 2002). Activation of VTA neurons initiates locomotor activity in order to obtain such primary rewards and this activation is associated with a high level of arousal – compounds which block the dopamine transporter, leading to enhanced dopaminergic tone in target regions, are potent wake-promoting substances (Wisor et al., 2001) . Initial recording studies searched for correlates of parkinsonian motor and cognitive deficits in dopamine neurons but failed to find clear covariations with arm and eye movements or with mnemonic or spatial components of delayed response tasks. By contrast, it was found that dopamine neurons were activated in a very distinctive manner by the rewarding characteristics of a wide range of somatosensory, visual, and auditory stimuli (Schultz, 2002).

About 75% of dopamine neurons show phasic activations when animals find hidden food during exploratory movements in the absence of other phasic stimuli, without being activated by the movement itself (Romo and Schultz, 1990). The remaining dopamine neurons do respond to any of the tested environmental stimuli. Dopamine neurons also are activated by a drop of liquid delivered into the mouth outside of any behavioral task or while learning such different paradigms as visual or auditory reaction time tasks, spatial delayed response or alternation, and visual methiocrimination, often in the same animal (Hollerman and Schultz, 1998). The reward responses occur independently of a learning context. Thus dopamine neurons do not appear to discriminate between different food objects and liquid rewards. However, their responses distinguish rewards from nonreward objects (Romo and Schultz, 1990). Only 14% of dopamine neurons show the phasic activations when primary aversive stimuli are presented, such as an air puff to the hand or hypertonic saline to the mouth, and most of the activated neurons respond also to rewards (Mirenowicz and Schultz, 1996). Though being nonnoxious, these stimuli are aversive - they disrupt behavior and induce active avoidance reactions. However, dopamine neurons are not entirely insensitive to aversive stimuli, as shown by slow depressions or occasional slow activations after pain pinch stimuli in anesthetized monkeys and by increased striatal dopamine release after electric shock and tail pinch in awake rats. This suggests that phasic responses of dopamine neurons preferentially report environmental stimuli with primary appetitive value, whereas aversive events may be signaled with a considerably slower time course (Schultz, 2002).

An important feature of dopamine responses is their dependency on event unpredictability. The activations following rewards do not occur when food and liquid rewards are preceded by phasic stimuli that have been conditioned to predict such rewards (Mirenowicz and Schultz, 1996). One crucial difference between learning and fully acquired behavior is the degree of reward unpredictability. Dopamine neurons are activated by rewards during the learning phase but stop responding after full acquisition of visual and audiatory reaction time tasks, spatial delayed response tasks, and simultaneous visual discriminations. The loss of response is not due to a developing general insensitivity to rewards, as activations following rewards delivered outside of tasks do not decrement during several months of experiments. The importance of unpredictability includes the time of reward, as demonstrated by transient activations following rewards that are suddenly delivered earlier or later than predicted (Hollerman and Schultz, 1998). Taken together, the occurrence of reward, including its time, must be unpredicted to activate dopamine neurons.

Dopamine neurons are depressed exactly at the time of the usual occurrence of reward when a fully predicted reward fails to occur, even in the absence of an immediately preceding stimulus. This is observed when animals fail to obtain reward because of erroneous behavior, when liquid flow is stopped by the experimenter despite correct behavior, or when a valve opens audibly without delivering liquid (Hollerman and Schultz, 1998). When reward delivery is delayed for 0.5 or 1.0 s, a depression of neuronal activity occurs at the regular time of the reward, and an activation follows the reward at the new time. Both responses occur only during a few repetitions until the new time of reward delivery becomes predicted again. By contrast, delivering reward earlier than habitual results in an activation at the new time of reward but fails to induce a depression at the habitual time. This suggests that unusually early reward delivery cancels the reward prediction for the habitual time. Thus dopamine neurons monitor both the occurrence and the time of reward. In the absence of stimuli immediately preceding the omitted reward, the depressions do not constitute a simple neuronal response but reflect an expectation process based on an internal clock tracking the precise time of predicted reward.

The characteristics of dopamine responses to reward-related stimuli are best illustrated in learning episodes during which rewards are particularly important for acquiring behavioral responses. The dopamine reward signal undergoes systematic changes during the progress of learning and occurs to the earliest phasic reward-related stimulus, this being either a primary reward or a reward-predicting stimulus (Mirenowicz and Schultz, 1996). During learning, novel, intrinsically neutral stimuli transiently induce responses that weaken soon and

disappear. Primary rewards occur unpredictably during initial pairing with such stimuli and elicit neuronal activations. With repeated pairing, rewards become predicted by conditioned stimuli. Activations after the reward decrease gradually and are transferred to the conditioned, reward-predicting stimulus. If, however, a predicted reward fails to occur because of an error of the animal, dopamine neurons are depressed at the time the reward would have occurred. During repeated learning of tasks or task components, the earliest conditioned stimuli activate dopamine neurons during all learning phases because of generalization to previously learned, similar stimuli, whereas subsequent conditioned stimuli and primary rewards activate dopamine neurons only transiently while they are uncertain and new contingencies are being established. Dopamine responses are elicited by three categories of stimuli. The first category comprises primary rewards and stimuli that have become valid reward predictors through repeated and contingent pairing with rewards. These stimuli form a common class of explicit reward-predicting stimuli, as primary rewards serve as predictors of vegetative rewarding effects. Effective stimuli apparently have an alerting component, as only stimuli with a clear onset are effective (Schultz, 2002).

Dopamine neurons show pure activations following explicit reward-predicting stimuli and are depressed when a predicted but omitted reward fails to occur. The second category comprises stimuli that elicit generalizing responses. These stimuli do not explicitly predict rewards but are effective because of their physical similarity to stimuli that have become explicit reward predictors through conditioning. These stimuli induce activations that are lower magnitude and engage fewer neurons, as compared with explicit reward-predicting stimuli. They are frequently followed by immediate depressions. Whereas the initial activation may constitute a generalized appetitive response that signals a possible reward, the subsequent depression may reflect the prediction of no reward in a general reward-predicting context and cancel the erroneous reward assumption. The lack of explicit reward prediction is suggested further by the presence of activation after primary reward and the absence of depression with no reward. Together with the responses to reward-predicting stimuli, it appears as if dopamine activations report an appetitive ‘‘tag’’affixed to stimuli that are related to rewards (Schultz, 2002).

The third category comprises novel or particularly salient stimuli that are not necessarily related to specific rewards. By eliciting behavioral orienting reactions, these stimuli are alerting and command attention. However, they also have motivating functions and can be rewarding. Novel stimuli are potentially appetitive. Novel or particularly salient stimuli induce activations that are frequently followed by depressions, similar to responses to generalizing stimuli. Thus the phasic responses of dopamine neurons report events with

positive and potentially positive motivating effects, such as primary rewards, reward-predicting stimuli, reward-resembling events, and alerting stimuli. However, they do not detect to a large extent events with negative motivating effects, such as aversive stimuli. (Schultz, 1998). Taken together, it appears that the processing of specific rewards for learning and maintaining approach behavior would profit strongly from a cooperation between dopamine neurons signaling the unpredicted occurrence or omission of reward and neurons in the other structures simultaneously, indicating the specific nature of the reward.

The functions of VTA GABAergic neurons are less studied; therefore, in last years it is becoming clear, that their contribution in reward processes is more complicated than just inhibition of DAergic neurons. It was demonstrated (Steffensen et al., 2001) that approximately 2–3 s before the rat nosepoked for the intracranial self-stimulation (ICSS) of medial forebrain bundle (MFB), the spontaneous activity of each neuron increased progressively reaching a peak around 1–2 s before the time of nosepoke for MFB ICSS. After MFB ICSS, the discharge activity was inhibited for around 2 s. On the contrary, before passive MFB stimulation, neuron firing was unaffected; however, similar to “active” MFB stimulation, decreased after stimulation. Interestingly, the pre-stimulation increase in VTA neuron discharge activity during MFB ICSS often peaked and terminated before the nosepoke for MFB stimulation, suggesting that these neurons may play a role in the attention to the possibility of brain stimulation reward. Such increase in activity of GABAergic VTA neurons during approach may reflect the rat's attention to the potential or predictability of the rewarding stimulus. Once behavioral responding for ICSS stabilized there was no adaptation of the GABAergic neurons to pre-stimulation increase or post-stimulation decrease in discharge activity either within a particular session or during any subsequent ICSS session. In this respect the pattern of activation of GABAergic neurons differs from those of DAergic neurons in VTA . Interestingly, this pattern parallels the lack of tolerance to the ICSS behavior itself (Steffensen et al., 2001). Heroin self-administration produces a pre-injection increase in VTA neuron activity and a post-injection inhibition of VTA neuron activity. A number of findings suggested that VTA non-DA neurons may regulate cortical arousal and psychomotor systems: VTA non-DA firing rates increase markedly during the onset of movement and decrease markedly with select anesthetics (Steffensen et al., 1998). In addition, the firing rate of VTA non-DA neurons decreased 42% during slow-wave sleep and increased 114% during REM sleep, relative to wakefulness. If an increase in the mesolimbic dopaminergic tone is important in brain-reward mechanisms (Wise and Rompre, 1989), it is reasonable that GABA inhibition of DA neurons by VTA non-DA neurons may be an

important mechanism of regulation. In support of this hypothesis, it has recently been demonstrated that self-administration of GABA antagonists into the VTA is blocked by D2 receptor antagonists (David et al., 1997). On the other hand, a role for DA in ICSS reward remains controversial as there is no DA release, or decreased DA release, during each operant response for ICSS, suggesting that DA is a neural substrate for novelty or reward expectation, rather than reward itself. The GABAergic VTA neurons could play a critical role in attention to rewarding stimuli, rather than as transducers of reward (Steffensen et al., 2001).

A large body of evidence indicates the existence of a fine balance between the ascending dopaminergic and descending GABAergic branches of the mesostriato-mesencephalic loop (Smith and Bolam, 1990). The lesion or pharmacological manipulation of either of these causes functional and morphological changes in the other; for example, the dopaminergic denervation of the striatum leads to a remodelling of postsynaptic neurons with an increase of GABAergic boutons and their synaptic contacts, and changes in GAD mRNA expression (Gonzalez-Hernandez et al., 2001). By contrast, the administration of GABAergic agents and the electrical stimulation of the GABAergic nigrostriatal feedback (Paladini et al., 1999), can produce both a direct inhibition and an indirect excitation of dopaminergic neurons.

6. Feeding.

6.1. Feeding is a natural reward.

Feeding provides substrate for energy metabolism, which is vital to the survival of every living creature and therefore is subject to intense regulation by brain homeostatic and hedonic systems. Mammalian brains have evolved several potent and interrelated neuronal systems that drive feeding behavior. One of the most potent drives for feeding is its rewarding nature. Any mammal will eat beyond its homeostatic needs if presented with highly palatable food, which illustrates the rewarding nature of this activity. What makes certain foods rewarding beyond their metabolic content?

First, to understand the rewarding nature of food, it is necessary to consider the various homeostatic and hedonic mechanisms that underlie regulation of feeding and make it such a rewarding experience.

6.2. Hypothalamus.

The hypothalamus is providing the suitable neurological substrate for coordinating the needs of the individual animal with dynamic changes in the environment. The adaptive response involves complex endocrine, autonomic and somatomotor mechanisms that must be integrated for these responses to give an optimal benefit to the animal. Moreover, these responses must be coordinated and subject to a hierarchy of homeostatic priorities, a process termed "motivational time-sharing". Hypothalamic circuits can be viewed as essentially consisting of sensory and motor components, with intrahypothalamic integrative circuits interposed between the sensory and the motor parts. The hypothalamus receives sensory information from visceral sensory regions such as the nucleus of the solitary tract and the subfornical organ as well as olfactory and other sensory information from limbic regions (in particular, amygdala). Superimposed on hypothalamic regions receiving these sensory pathways are projections from the brain stem reticular nuclei that may effect a more general arousal. With respect to motor pathways, the hypothalamus sends direct and massive projections to preganglionic autonomic regions and all parts of the pituitary gland, and also to regions that mediate somatomotor responses. The hypothalamus is best regarded as 3 distinct longitudinal zones – periventricular, containing most of the neurons that project to the pituitary, the medial zone, consisting of distinct cell groups that receive their major inputs from septum and amygdala, and the lateral zone. Neurons located in the lateral zone of the hypothalamus are distributed among the fibers of the medial forebrain bundle and can be viewed as a bed nucleus for this complex system (Simerly, 1995).

For many years, descriptions of the brain mechanisms for regulating the homeostatic drive to eat were fixated on a model of two opposing cell groups, the lateral hypothalamus driving feeding and the ventromedial nucleus inhibiting it to cause satiety. This model was derived originally from clinical observations in patients with Frolich's syndrome (1901): pituitary tumors associated with excessive subcutaneous fat. The role of hypothalamus in regulating food intake was established in experiments of Hetherington and Ranson (1940). They produced bilateral electrolytic lesions in the hypothalamus of rats and found that a condition of marked adiposity characterized by as much as a doubling of body weight and an increase of extractable body lipids was produced by lesions of the dorsomedial and ventromedial hypothalamic nuclei, the arcuate nucleus, the fornix, the portion of the lateral hypothalamic area ventral to it and probably also the ventral premamillary nuclei. Conversely, lesions in adjacent lateral hypothalamus could lead to decreased food intake. Further experiments (Anand, 1951) demonstrated that the lesions of the lateral hypothalamus at the level adjacent to the ventromedial nucleus caused loss of feeding and even a death by starvation. Later,

several groups pointed out that the lateral hypothalamic lesions that cause hypophagia interrupt the ascending nigrostriatal bundle, but others found that lateral hypothalamic lesions produced by kainic acid resulted in hypophagia, even when the ascending dopaminergic system was not damaged. Electrical self-stimulation studies of the LHA demonstrate that the LHA participates in a dopaminergic transmission dependent reward system that is markedly facilitated by food deprivation (Carr and Wolinsky, 1993). Further neuroanatomical studies showed a lateral hypothalamic cell system with direct projections both to the cerebral cortex and to the autonomic and motor systems of the brainstem and the spinal cord. This extensive system was predicted to support a lateral hypothalamic phagic function. This prediction was confirmed by the subsequent discovery of two peptides, which are expressed exclusively in the perifornical region and lateral hypothalamic area - orexins, also called hypocretins, and melanin-concentrating hormone.

7. Neuropeptides involved in the regulation of food intake.

7.1.Orexins

Orexin A is a 33 amino-acid peptide (3562Da). Orexin B (2937Da) has 46% (13/28) amino acid identity to the orexin A sequence. A single gene composed of two exons and an intervening intron encodes the orexin neuropeptides. The structure is conserved within rodent and human genomes. They were found simultaneously by two groups and called “orexins” reflecting their role in food intake (Sakurai et al., 1998) and “hypocretins” because they share substantial amino acid identities with the gut hormone secretin (de Lecea et al., 1998).

7.1.1. Orexin receptors.

Sakurai et al (Sakurai et al., 1998) identified two orexin receptor subtypes, named orexin-1 (Ox1R) and orexin-2 receptors (Ox2R) that are structurally similar to other G-protein-coupled neuropeptide receptors. Ox2R has 64% amino acid identity with OX1R. The Ox1R has a higher affinity for Or-A compared with Or-B (50% inhibitory concentration – IC50 = 20 nM and 250 nM, respectively). In contrast, both orexins bind the Ox2R with equal affinity (IC50=20nM). Therefore, it appears that Ox1R is moderately selective for OrA, whereas Ox2R is a nonselective receptor for both orexins. Evidence from receptor-transfected cell lines and isolated receptor-expressing hypothalamic neurons suggest that OX1R is coupled exclusively to the Gq subclass of heterotrimeric G proteins, whereas Ox2R may couple to Gi/o and/or Gq (Sakurai et al., 1998).

7.1.2. Neuroanatomy of the orexin system

The orexin-producing group of cells is restricted to the lateral hypothalamic and perifornical areas, but their projections reach the entire brain (de Lecea et al., 1998, Peyron et al., 1998). Particularly abundant projections are found in the cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, diagonal band of Broca, bed nucleus of the stria terminalis, thalamus, anterior and posterior hypothalamus, midbrain, brainstem and spinal cord. Orexin immunoreactivity is also reported in the enteric nervous system, pancreas and testes. Orexin receptors are expressed in a pattern consistent with orexin projections but often have a differential distribution (Marcus et al., 2001). Ox1R mRNA is highly expressed in the prefrontal cortex, hippocampus, paraventricular thalamus, ventromedial hypothalamus (VMH), arcuate nucleus (ARC), dorsal raphe nucleus (DR) and locus coeruleus (LC). Ox2R mRNA is found in the cerebral cortex, medial thalamic groups, DR and many hypothalamic nuclei, including the tuberomamillary nucleus (TM), dorsomedial hypothalamus (DMH), paraventricular hypothalamic nucleus (PVN) and ventral premamillary nuclei. The study of the anatomical relationships between orexin and dopamine neurons in rats revealed a large number of retrogradely-labeled cells that formed a band extending from the medial perifornical area arching dorsally over the fornix and then ventrolaterally into the lateral hypothalamus (Fadel and Deutch, 2002). 20% of these cells express orexin A-like immunoreactivity. Axons that were anterogradely labeled from the lateral hypothalamus were seen throughout the ventral tegmental area, and laid in close proximity to the dendrites and somata of dopamine neurons. Dopamine and orexin fibers were found to codistribute in the medial prefrontal cortex; orexin fibers were present in lower density in the medial shell of the nucleus accumbens, and the central and posterior basolateral nuclei of the amygdala.

Orexin neurons also express mRNA for the orexigenic opioid dynorphin and the secretory marker secretogranin II, and the biosynthesis of these three peptides may be similarly regulated. Galanin, another appetite-stimulating neuropeptide, has been identified in orexin neurons. MCH neurons are also found exclusively in the LHA and project diffusely throughout the entire CNS, however, they never coexpress orexins (Broberger et al., 1998).

7.1.3. Effect of orexins on feeding.

Acute injection of OrA into the lateral ventricles of fed rats during the early light phase, dose-dependently and significantly stimulates food consumption (Yamanaka et al., 1999). Similar experiments with OrB were inconsistent, but positive studies revealed that the feeding effects

were shorter than those of OrA, that can be explained by longer resistance of OrA to peptidases due to its disulfide bonds or by existence of subsets of Ox1R and Ox2R-mediated feeding pathways. OrA is significantly less potent in stimulating food consumption than NPY, but its duration of action is apparently longer than that of NPY (Sakurai et al., 1998), and the magnitude of the maximal effect of orexins is similar to that of other appetite stimulating peptides, such as MCH and galanin (Edwards et al., 1999). The central administration of an anti-orexin antibody significantly and dose-dependently suppresses spontaneous feeding in fasted rats. A selective Or1R antagonist inhibits natural feeding, feeding stimulated by fasting or i.c.v. injection of OrA. OrA dose-dependently increases gastric acid secretion when given centrally. The feeding response to acute i.c.v. injection of orexinA is dependent on the time of the day: there was no effect of orexins at the beginning of the dark phase when normal food intake is at its highest, but in the early light phase and 6hr into the dark phase the largest Or-induced increase of food intake was observed (Haynes et al., 1999). One can suggest that, at the beginning of the dark cycle, when the rat is maximally aroused, orexin-stimulated pathways are already activated and therefore unresponsive to additional stimulation by orexins. In more nuclei-specific studies it was found that OrA stimulates feeding when injected into the PVN, DMH, LHA and perifornical area of the rat. Parallel experiments using OrB showed no effect on feeding. Several recent studies have hypothesized a fundamental role of orexin in coordinating endocrine and autonomic responses to falling glucose levels. For example, hypoglycemia induces Fos expression in orexin neurons and increases orexin mRNA expression (Moriguchi et al., 1999). Hence, orexin neurons may represent one of the populations of “glucose-inhibited” neurons in the lateral hypothalamus that respond to physiological falls in glucose levels with an increase in activity.

Centrally injected orexins increase drinking at doses similar to those that evoke feeding (Kunii et al., 1999). This effect is similar in potency to angiotensin II but orexin-stimulated water intake is longer-lasting. These effects are in keeping with the orexin innervation of the subfornical organ and the area postrema, regions known to be inportant in fluid homeostasis. In addition, preprooprexin mRNA levels are upregulated during fluid deprivation.

7.1.4. Orexins promote arousal

More than 70 years ago, von Economo predicted a wake-promoting area in the posterior hypothalamus and a sleep-promoting region in the preoptic area. Recent studies have dramatically confirmed these predictions. The ventrolateral preoptic nucleus contains GABAergic and galaninergic neurons that are active during sleep and are necessary for

normal sleep. The posterior lateral hypothalamus contains the orexin neurons that appear to be crucial for maintaining normal wakefulness. Sleep studies in rats found that Fos immunoreactivity in orexin neurons is positively correlated with wakefulness and negatively correlated with the amount of non-REM and REM sleep, suggesting that these neurons are waking-active. They further demonstrated that orexin neuronal activity is under strong circadian control since the temporal relationship with wakefulness and the usual onset of the dark phase is preserved even under conditions of constant darkness. Fos in orexin neurons is also increased after mild sleep deprivation and administration of the wake-promoting drugs modafinil and amphetamine. I.c.v. administration of OrA in rodents dose-dependently increases wakefulness and supressess non-REM and REM sleep. Orexin injection also causes behavioural changes typical for the aroused state, including increased locomotor activity, rearing, grooming and searching behaviour (Hagan et al., 1999). In vitro orexins excite all monoaminergic cell groups, which together constitute an ascending arousal system (Saper et al., 2001): they depolarize serotonergic cells in dorsal raphe and elicit sodium-dependent action potentials (Brown et al., 2001a; Brown et al., 2002), noradrenergic neurons in locus coeruleus (Hagan et al., 1999) and histaminergic neurons in the tuberomamillary nucleus (Eriksson et al., 2001).

The importance of orexin signalling in wakefulness is emphasized by the fact that mutation in the Or2R gene (Mignot, 1998) or loss of orexin neurons (Nishino et al., 2000) cause the disease narcolepsy which is characterized by excessive daytime sleepiness, cataplexy – sporadic loss of muscle tone, and spontaneous occurrence of REM-associated phenomena during wakefulness. Cataplexy is often provoked by strong positive emotions (for example, in dogs food-elicited narcolepsy is observed). Orexin knockout mice (Chemelli et al., 1999) or rats with selective saporin lesions of orexin 2 neurons (Gerashchenko et al., 2001) have a narcoleptic phenotype as well. The hyperlocomotion and stereotypy induced by intracerebroventricular orexin application are blocked by dopamine receptor antagonists, and orexins increase intracellular calcium in acutely isolated A10 dopamine neurons (Nakamura et al., 2000). The excessive daytime sleepiness of narcoleptics is currently treated with amphetamine like compounds, which enhance extracellular dopaminergic levels (Nishino and Mignot, 1997; Wisor et al., 2001; Nakamura et al., 2000). Furthermore, application of dopamine D_2 receptor agonists either systemically or locally into the VTA exacerbates cataplexy (periods of muscle weakness triggered by emotional stimuli in narcoleptics), whereas D_2 receptor antagonists have the opposite effect (Reid et al., 1996; Okura et al., 2000). Thus, the dopamine system could mediate some actions of the orexins. Another

neuropeptide, which is exclusively expressed in the same area, is melanin-concentrating hormone.

7.2. Melanin-concentrating hormone (MCH).

The neurons containing MCH and orexins are intermingled but the peptides are not coexpressed (Broberger et al., 1998); both cell groups have very similar and enormously wide-ranging projections (Bittencourt et al., 1992, Peyron et al., 1998). The receptors for both peptides are similarly widely distributed (Kilduff and de Lecea, 2001). Intracerebroventricular injections of MCH increase food intake in the rat, and MCH mRNA levels are increased by food deprivation. Leptin-deficient *ob/ob* mice have elevated levels of MCH mRNA (Qu et al., 1996). Most persuasively, $MCH^{-/-}$ mice are hypophagic and lean, and mice that overexpress MCH are obese and hyperleptinemic (Ludwig et al., 2001). The MCH-1 receptor antagonist SNAP-7941 inhibits food intake stimulated by central administration of MCH, reduces consumption of palatable food, and, after chronic administration to rats with diet-induced obesity, results in a marked, sustained decrease in body weight. It also produces effects similar to clinically used antidepressants and anxiolytics in three animal models of depression/anxiety: the rat forced-swim test, rat social interaction and guinea pig maternal-separation vocalization tests (Borowsky et al., 2002). MCH and orexin neurons project to structures known to participate in feeding (Bittencourt et al., 1992),including the brainstem motor systems that support behaviors like chewing, licking, and swallowing, served by motor neurons in the trigeminal, facial, and hypoglossal nuclei, as well as the reticular areas that surround them and which constitute pattern generators for these behaviors (Lund et al., 1998). The MCH and orexin neurons also innervate the sympathetic and parasympathetic preganglionic nuclei in the medulla and the spinal cord. These sites are critical for promoting salivation, esophageal and gastric motility, gastric acid secretion, and regulation of the secretion of pancreatic hormones, including insulin and glucagons. A third important target of the MCH and orexin neurons are the monoaminergic nuclei. In addition, the MCH and orexin neurons innervate the entire cerebral cortex. Thus, they are able to enhance arousal and locomotor activity and by this way increase the likelihood that an animal will encounter food, while priming consumatory pattern generators to lower the threshold for their activation if a likely food source is identified (Saper et al., 2002).

One of the few sites where the projections of the MCH and orexin neurons differ is the nucleus accumbens, which receives primarily an MCH input and bears mainly MCH receptors (Peyron et al., 1998, Bittencourt et al., 1992). By enhancing activity in the nucleus

accumbens, the MCH neurons may enhance the hedonic value of food, and by receiving inputs from the nucleus accumbens, they may participate in a self-reinforcing circuit thatsupports feeding.

7.3. Cocaine and amphetamine regulated transcript (CART)

Recent evidence indicates the expression of neuropeptides that decrease food intake and body weight in the lateral hypothalamic area, and this may play a counter-regulatory role, opposing the systems that increase food intake. One such peptide, CART (cocaine and amphetamine regulated transcript) is expressed in the lateral hypothalamic area and decreases food intake when centrally administered. It has been reported that CART peptides colocalize with melanin-concentrating hormone (MCH) in the lateral hypothalamus and that the expression of both peptides is regulated by leptin (Kristensen et al., 1998). CART was originally discovered as a novel up-regulated mRNA in a drug abuse paradigm (Douglass et al., 1995). Soon after this novel family of neuropeptides was identified, CART peptides were found to exert effects on food intake after intracerebroventricular injection into the rat brain (Kristensen et al., 1998). CART peptide-containing neurons are abundant in the core and shell of the nucleus accumbens, whereas the substantia nigra and ventral tegmental area contain CART-immunoreactive axons and terminals (Smith et al., 1999). The pattern of distribution of CART peptide-immunoreactive fibers in the ventral midbrain is strikingly similar to that displayed by dynorphin-containing afferents, which, for the most, arise from the NAc. Together, these observations suggest that CART peptides and dynorphin may coexist in a descending striato-mesencephalic projection. The lateral hypothalamus is one of the primary sources of inputs to the rat VTA (Zahm et al., 2001). Intra-VTA injections of CART produces the psychomotor stimulant-like effects of increased locomotor activity and conditioned place preference (Kimmel et al., 2000). A series of experiments was performed (Dallvechia-Adams et al., 2002) to elucidate the source, synaptic connectivity, and neurochemical content of CART peptide-immunoreactive (CARTir) terminals in the rat VTA. Double-labelling immunofluorescence revealed that approximately 15% of CARTir terminals in the VTA contain melanin-concentrating hormone (MCH). Furthermore, CART peptides were also found colocalized with GABA and, to a small extent, with dynorphin in nerve terminals in both the VTA and SN. In the VTA, CARTir terminals form both symmetric and asymmetric synapses onto dopaminergic and nondopaminergic distal dendrites, suggesting that various sources contribute to this innervation. About 30% of CARTir terminals in the VTA and only 15% in the SN appose or form synaptic contact with DA neurons, which support the data

showing that GABAergic basal ganglia output neurons in the substantia nigra pars reticulata (SNr) receive strong CARTir input from the accumbens core. Results of these studies suggest that CART-influenced feeding and locomotion may be mediated by direct and/or indirect modulation of VTA dopaminergic neuronal activity (Dallvechia-Adams et al., 2002).

In dehydrated rats mRNA encoding the precursor for corticotropin-releasing factor (CRF) is increased in a restricted region of the lateral hypothalamic area (Richard et al., 2002). As CRF is an anorectic peptide, it is possible that lateral hypothalamic area neurons are influenced by physiological stimuli to integrate body weight and food intake. On the other hand, in contrast to orexins and MCH, CART and CRF are produced not exclusively in LHA, and their anorectic actions could be mediated through other sites –for example, nearly all CART neurons in the arcuate and retrochiasmatic area coexpress proopiomelanocortin, and its derivate, alpha-MSH is a strong anorectic (see below). There is a number of other mechanisms that regulate satiety system in the brain. The ventromedial and arcuate nuclei have long been implicated in the integration of circulating autonomic signals producing satiety. Lesions of these nuclei cause overeating and obesity. They lie directly over the median eminence, a region where the blood-brain barrier is disrupted and an exchange of signalling molecules with the bloodstream takes place, where circulating hormones, such as leptin, can enter the brain.

7.4. Leptin

Zhang and colleagues identified the mutation resulting in obesity in the extensively studied *ob/ob* mouse (Zhang et al., 1994). They found that the *ob* gene encoded a hormone that was made and secreted by white adipose tissue. They called the OB protein "leptin," from the Greek root leptos "thin". A number of subsequent studies demonstrated that leptin levels in the blood increase when animals are fed and fall when animals are deprived of food (Frederich et al., 1995). Moreover, it quickly became clear that leptin replacement in *ob/ ob* mice corrected their characteristic behavioral, neuroendocrine, and autonomic abnormalities (Campfield et al., 1995). Then it was demonstrated that the *db/db* mouse had a mutation in the long form of the leptin receptor (Chen et al., 1996). Although the high leptin levels in obese humans indicate that the presence of excess leptin is not a strong inhibitor of feeding, the ravenous appetite in humans or animals that lack the leptin receptor or ligand indicates that its absence is a strong stimulus to induce feeding. The leptin receptor is a type 1 cytokine receptor exerting its effects by activating the janus-kinase/STAT-3 pathway. The long form of

the leptin receptor is present in several brain sites, with the most dense expression being found in the ventral basal hypothalamus, especially the arcuate, ventromedial, dorsomedial, and ventral premamalliary nuclei in the hypothalamus (Saper et al., 2002). It is suggested that leptin could alter food intake partly by reducing the appetitive value of food. It is known that perifornical self-stimulation is modulated by chronic food restriction; there are sites where rate-frequency function shifts leftward after chronic food restriction, towards weaker stimulation strength. Leptin decreases the effectiveness of the rewarding stimulation namely in these sites, but increases the effectiveness of reward stimulation in LH sites which are unresponsive to food restriction. Thus, leptin could make complementary contribution to energy balance by reducing food reward while enhancing the value of behaviours incompatible with feeding (Fulton et al., 2000). Leptin also inhibits responses of sweet-sensitive taste cells in the tongue (Kawai et al., 2000), suggesting that it may mediate the hedonic value of food reward beginning at the most peripheral level. Leptin attenuates the ability of fasting to increase heroin-seeking behavior (Shalev et al., 2001). However, the specific leptin-responsive cell groups mediating these effects are not known.

The neurons that bear high levels of leptin receptors cluster around the median eminence. Studies using circulating radiolabeled leptin indicate that it binds to these regions in rat brain. Although circulating leptin binds primarily to these hypothalamic cell groups, leptin receptors are expressed (albeit at lower levels) in several extrahypothalamic sites, including the brainstem. The physiological significance of leptin receptor expression in these sites is not as well established, but it is likely that they contribute to the diverse effects of leptin and leptin deficiency. Administration of exogenous leptin activates STAT-3 translocation, phosphorylation of phosphatidylinositol-3-OH-kinase (PI3-K), and the expression of several genes, including SOCS-3 and c-*fos* in neurons in hypothalamic and brainstem cell groups that bear leptin receptors (Elmquist et al., 1998). Leptin-responsive neurons in the arcuate nucleus include those containing neuropeptide Y (NPY) and agouti-related protein (AgRP) in the medial part of the nucleus and those containing pro-opiomelanocortin (POMC) and its derivatives, including α-melanocyte stimulating hormone (α-MSH), as well as cocaine and amphetamine-related transcript (CART). The POMC/CART neurons show both Fos and SOCS-3 expression after leptin, suggesting that they have been activated. This is supported by the observation that leptin directly depolarizes POMC neurons in hypothalamic slices (Cowley et al., 2001). POMC neurons innervate the paraventricular nucleus, as well as the melanin concentrating hormone (MCH) and orexin/hypocretin cells in the LHA, and sympathetic preganglionic neurons in the spinal cord. The NPY/AgRP neurons express

SOCS-3 but not Fos after leptin administration, suggesting that they are inhibited (Cowley et al., 2001). They innervate many of the same targets as the POMC/CART cells. In the hypothalamus, α-MSH contained in the POMC terminals is an agonist at melanocortin-4 receptors, whereas AgRP is a peptide antagonist at the same receptors. Thus, the NPY/AgRP neurons are thought to constitute a potent feeding system that is actively opposed by the POMC/CART satiety system, and leptin apparently can push the balance toward the latter (Saper et al., 2002).

7.5. Neuropeptide Y (NPY)

NPY is a widely distributed CNS neuropeptide that has long been considered a major regulator of feeding. Injections of NPY increase feeding, reduce the latency to feeding initiation and increase meal size without substantially altering meal frequency (Gehlert, 1999). Rats administrated repeated doses of NPY exhibit a consistent increase in feeding without developing tolerance to the effects of the peptide. In rats, the most sensitive area to NPY is the perifornical region (Stanley et al., 1993); that is interesting in the context of NPY and orexin interaction. Administration of NPY into the region of the paraventricular nucleus also increases feeding. Neurons expressing NPY in the medial part of the arcuate nucleus project to the paraventricular nucleus. In fasted mice, as well as in *ob/ob* and *db/db* mice, NPY mRNA is elevated in the arcuate nucleus. Leptin administration blunts this overexpression. Fos expression in NPY neurons of the arcuate nucleus is induced by i.c.v. injection of orexin, suggesting that orexin-stimulated feeding may occur through the NPY pathways (Elias et al., 1998); however, orexin-stimulated feeding is only partially inhibited by pretreatment with NPY antagonists, suggesting that independent pathways for these peptides exist (Yamanaka et al., 2000). In obese rats NPY mRNA expression was tenfold increased whereas expressions of the Y1 and Y5 receptors were decreased by 25% and 50%, respectively. At the same time, their prepro-orexin mRNA expression was more than twofold decreased and expressions of their orexin receptors 1 and 2 mRNA were five- and fourfold increased, respectively. An inverse phenomenon was thus noted between the two peptides: for NPY, increased levels and downregulation of receptors; and for orexins, diminished levels with upregulation of receptors (Beck et al., 2001). The reasons for these changes might be linked to the absence of leptin signaling as similar profiles are found in the ob/ob mice. For orexins, other factors such as hyperglycemia might be involved.

Neuropeptide Y-immunoreactive neurons in the rat striatum receive synaptic inputs from dopaminergic axon terminals. Double immunocytochemical study revealed that NPY-

immunoreactive neurons receive synaptic inputs from catecholaminergic axon terminals in the rat neostriatum. Tyrosine hydroxylase-immunoreactive axons were found to be in synaptic contact with the somas and proximal dendrites of NPY-immunoreactive neostriatal neurons (Kubota et al., 1988). Thus nigrostriatal dopaminergic neurons may monosynaptically influence striatal NPY neurons. The NPY system may be regulated by selective activity at postsynaptic or presynaptic dopamine receptors. Treatment with the D1 receptor agonist, SKF 38393, decreased, while that with the D1 receptor antagonist, SCH 23390, increased NPY-like immunoreactivity in the globus pallidus and several regions within the caudate-putamen. SCH 23390 did not change accumbens NPY-like immunoreactivity levels but SKF 38393 increased NPY-like immunoreactivity levels in anterior and decreased NPY-like immunoreactivity levels in the posterior nucleus accumbens. Reductions in NPY-like immunoreactivity content occurred in response to administrations of both D2 receptor agonist, quinpirole, or antagonist, sulpiride, in all identified regions of each structure at some time point (Midgley et al., 1994). The administration of stimulant drugs which increase dopaminergic tone - methamphetamine and cocaine- markedly reduced the striatal content of NPY-like immunoreactivity, but had no effect on NPY in the globus pallidus or the substantia nigra. SCH-23390 blocked this effect suggesting that NPY levels throughout the nucleus accumbens and the caudate are regulated through D1 pathways (Westwood and Hanson, 1999).Five receptors for the PP-fold family of peptides exist. They are members of the seven transmembrane domain-G-protein coupled receptor family and coupled to Gi, mediating an inhibition of adenylyl cyclase. The Y1 receptor is considered to be postsynaptic and mediates many of the known actions of NPY in periphery. Y5 has the highest level in the brain. The feeding induced by exogenous NPY requires both Y5 and Y1 receptors to obtain the full response(Gehlert, 1999). Surprisingly, deletion of NPY or NPY receptor genes does not decrease food intake, body weight and response to starvation. NPY neurons project to CRF neurons and there is evidence suggesting that CRF and NPY exert opposite effects on eating behavior in the hypothalamus: NPY-induced feeding is significantly increased after blocking of CRF receptors (Heinrichs et al., 1993).Corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) produce reciprocal effects on anxiety in the amygdala cells (Sheriff et al., 2001). The molecular mechanisms of possible CRF-NPY interactions in regulating anxiety behavior are not known. In the central nervous system, the action of NPY leads to inhibition of cAMP production while CRF is known to stimulate levels of cAMP in the brain. Consequently, NPY may antagonize anxiety-like behavior by counter-regulating CRF-stimulated cAMP accumulation and activation of the protein kinase A pathway. These

findings suggest that CRF and NPY may counter-regulate each other in amygdalar neurons via reciprocal effects on the protein kinase A pathway.

7.6. Corticotropin-releasing factor (CRF)

Members of the CRF family of peptides are capable of strong anorectic and thermogenic actions that appear coordinated to maximize energy losses. The mammalian CRF system consists of at least two different CRF receptor subtypes, a CRF-binding protein and endogenous CRF receptor ligands such as the urocortins. CRF is a 41-amino acid peptide abundantly expressed in the paraventricular hypothalamic nucleus neurons that project to the median eminence to stimulate the secretion of adrenocorticotropic hormone. CRF represents the major controller of the basal and stress-induced activation of the pituitary–adrenal axis, which is in keeping with the observation that the only remarkable phenotype of the CRF knockout mouse is a pituitary–adrenal hyporeactivity (Contarino et al., 1999). CRF is also widely expressed throughout the brain (Merchenthaler et al., 1982) and in peripheral tissues. In the mammalian brain, it is significantly expressed in hypothalamic and extrahypothalamic regions, including the olfactory bulb, bed nucleus of the stria terminalis, medial preoptic area, paraventricular hypothalamic nucleus, lateral hypothalamus, central nucleus of amygdala, geniculate nucleus, Barington's nucleus, dorsal motor complex and inferior olive. The broad distribution of CRF neurons conforms to the many expected functions of the peptide (Turnbull and Rivier, 1997). When injected centrally, CRF evokes autonomic responses, widespread arousal and anxiety-like behaviors. It activates and inhibits the sympathetic and parasympathetic branches of the autonomic nervous system. CRF induces anorexia (Heinrichs and Richard, 1999). A decrease of CRF concentration has been reported in patients with Parkinson's disease and CRF immunoreactivity is altered in MPTP-treated rats (an animal model of parkinsonism induced by MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)(Huang and Lee, 1995).The CRF_1 receptor and the CRF_2 receptor together with the newly discovered non-mammalian CRF_3 receptor are G-protein-coupled receptor types that mediate the effects of the CRF family of peptides. In addition to binding to two receptors, CRF and its related peptides also bind to the CRF-binding protein (Richard et al., 2002). The CRF receptor 1 binds with high affinity CRF and urocortin as well as urotensin I. The CRF_1 receptor mRNA is broadly distributed in the brain with high densities of expression observed in cortical, hypothalamic, limbic and cerebellar regions. Within the paraventricular hypothalamic nucleus, CRF_1 receptor mRNA is not detected under basal conditions but can be acutely induced by stressful stimuli. Richard et al. have emphasized the involvement of the

CRF_1 receptor in the CRF-anxiogenic and anorexic effects of CRF (Richard et al., 2002). On the other hand, urocortins II and III, selective ligands for the CRF_2 receptor, have been described as `stress-coping' peptides (Hsu and Hsueh, 2001). Both peptides are capable of reducing anxiety, blood pressure and arousal. CRF also decreases meal size in rats with a null mutation of the leptin receptor, suggesting the independence of CRF effects from leptin (Kochavi et al., 2001).

7.7. Ghrelin

Another nutritionally regulated hormone that may act in concert with leptin, is ghrelin. (Tschop et al., 2000). Ghrelin has recently been found as an endogenous ligand of the growth hormone releasing peptides (GHRPs) receptor. This hormone is synthesized in the stomach, and its levels increase during food deprivation in animals (Kojima et al., 1999) and peak prior to meals in humans. Rising ghrelin levels in concert with falling leptin levels may serve as a critical signal to induce hunger during fasting. The interaction of these two opposing nutritionally regulated hormones is currently an area of active investigation. The site of action for ghrelin on feeding is thought to be the hypothalamus, where ghrelin receptors are found in the ventromedial and arcuate nuclei (Saper et al., 2002), but ghrelin receptors are also located in other sites, and the actual mechanisms of action remain to be established. In the arcuate nucleus, NPY/AgRP neurons express ghrelin receptors (Willesen et al., 1999), and ghrelin induces immediate early gene expression in NPY neurons. NPY antagonists blunt the actions of ghrelin to induce feeding (Tschop et al., 2000). However, $NPY^{-/-}$ mice still respond to ghrelin, although some of that response could be due to AgRP release from the same neurons. The role of ghrelin in motivating feeding is underscored by recent studies in humans. Patients who had lost an average of 17% of their body weight via enforced dieting (and who typically regain most of the weight when the diet is ended) have significantly increased ghrelin levels with exaggerated peaks in pre-meal ghrelin secretion compared to obese controls. On the other hand, morbidly obese patients who underwent a gastric bypass operation and who lost 36% of their body weight have greatly reduced ghrelin levels, with loss of pre-meal peaks in ghrelin secretion, and are much more successful at maintaining their weight loss (Saper et al., 2002). Conversely, patients with the Prader-Willi syndrome, who have profound obesity and voracious uncontrollable appetite, have remarkably high levels of ghrelin when compared to other obese individuals (Cummings et al., 2002). Taken together, these observations suggest that ghrelin plays a key role in the motivation for feeding, but further work is needed to determine its mechanisms of action and the CNS circuits through which it exerts its effects.

7.8. Melanocortin System

The leptin-responsive POMC/CART neurons in the arcuate nucleus cause anorexia and weight reduction. This effect is thought to be mediated by actions of α-MSH on melanocortin 3 and 4 receptors (MC3-Rs, MC4-Rs) (Saper et al., 2002). Leptin-induced decrease in food intake is inhibited by pretreatment with the selective MC4 antagonist, HS014 (Kask et al., 1998). Because AgRP is an endogenous antagonist of α-MSH at these same receptors, intracerebroventricular administration of AgRP or overexpression of the AgRP gene greatly increases food intake and body weight (Ollmann et al., 1997). The MC4-R is expressed predominantly in the CNS, and MC4-R blockade causes an obesity phenotype. *MC4-R*$^{-/-}$ mice, humans and mice lacking POMC are obese (Yaswen et al., 1999). Mutations that cause loss of function of the MC4-R or POMC genes in humans produce obesity and insulin resistance, and up to 5% of morbidly obese humans may have spontaneous mutations in the MC4-R gene. In addition, the melanocortin system may mediate the anorexia and weight loss characteristic of illness and cancer cachexia models. For example, *MC4-R*$^{-/-}$ mice are resistant to a variety of models of cancer and cytokine-induced anorexia, and AgRP is effective at increasing food intake in anorexia models. The main effect of α-MSH on many PVH neurons may be mediated by presynaptic MC4-Rs expressed by local GABAergic interneurons (Cowley et al., 1999). MC4-R mRNA is expressed in several hypothalamic sites involved in regulating feeding, including the PVH, LHA, and arcuate nucleus (Mountjoy et al., 1994). In the perifornical part of the LHA, which contains orexin and MCH neurons, only a small subset of α-MSH terminals end upon these cell types (Elias et al., 1998). However, if MC4-Rs were expressed by local interneurons that provide inputs to the MCH and orexin neurons, the relationship could only be discerned by intracellular electrophysiology or electron microscopy. MC4-R mRNA is expressed in several extrahypothalamic regions of the brain thought to contribute to the regulation of feeding behavior. The nucleus accumbens (NAc) expresses MC4-R mRNA (Mountjoy et al., 1994) and innervates the LHA (Kirouac and Ganguly, 1995). The predominant neurons in the NAc that project to the LHA are GABAergic, and these are thought to contribute to the regulation of food intake (Stratford and Kelley, 1997). Orexinergic effects of a single injection of AgRP may persist for up to 6 days and are accompanied by a distinct pattern of Fos expression in the NAc. Thus, the melanocortin system may contribute not only to the homeostatic control of feeding but also to

its hedonic aspects via the MC4-R input to the NAc (Saper et al., 2002). There is a number of intriguing findings concerning the functional antagonism of opiates and melanocortin peptides: administration of melanocortins attenuates the acquisition of heroin self-administration and even inhibits the development of tolerance and physical dependence to opiates. Several studies demonstrated that melanocortins reduce the analgesic effect of morphine; they can also induce an opiate-withdrawal-like syndrome in drug-naive animals. Electrophysiological studies have indicated that melanocortins block morphine-induced depression of evoked potentials in lumbar ventral root in cats. On the other hand, morphine down-regulates the expression of POMC mRNA in the hypothalamus and MC4R mRNA expression in ventral and dorsal striatum but not in hypothalamus, frontal cortex, ventral tegmentum and substantia nigra (Alvaro et al., 1997). A recent study also suggests that the effects of drugs that act on the serotoninergic system may be mediated at least in part via the MC4-R (Heisler et al., 2002). d-Fenfluramine, which is widely used as part of the Phen/Fen combination to cause weight loss in humans, directly activates POMC neurons, probably via a serotonin 2c receptor mechanism. MC4-R antagonism blunts the anorectic actions of d-Fen. Thus, the MC4-R may play a key role in alterations of feeding and body weight in patients taking serotonin reuptake inhibitors for depression and may be involved in the weight loss that is typically seen in a variety of psychiatric disorders that are thought to affect the serotonin system, including depression and anorexia nervosa (Saper et al., 2002). Intra -VTA administration of α-MSH induces a significant increase in dopamine and DOPAC levels in the nucleus accumbens (Lindblom et al., 2001)

8. Mechanisms that regulate food intake

8.1. Gustatory Mechanisms

One unique quality of food reward is its strong modulation by palatability cues, particularly taste and smell. Animals will consume sweet and salty substances past the need for homeostatic repletion, while even food-deprived animals will avoid substances that are highly sour or bitter. These behaviors are of considerable adaptive value, as bitter tastes are often associated with toxic alkaloids, while the acidity of many sour substances can indicate spoilage or unripeness, and sweet and salty tastes indicate nutrients that are highly important for survival. The value of a flavor is also potently modified by postingestional cues. For example, administration of lithium chloride (which induces a feeling of illness) after an animal has tasted a novel flavor results in one-trial learning of a conditioned taste aversion to that flavor, even the one that would otherwise be highly preferred. Conditioned taste aversion

is also adaptive, as it allows animals to avoid foods that have made them sick in the past. It suggests, also, that there must be a potent circuitry by which the gustatory hedonic value of flavors can be modified (Saper et al., 2002). There have recently been substantial advances in our understanding of taste discrimination at the molecular level. Genes have been recently identified for a large number of taste receptors, including families of receptors
recognizing the classic four tastes (sweet, salty, sour, bitter) (Lindemann, 2001), as well as glutamate and possibly other amino acids (Nelson et al., 2001). However, it is not known how taste information ultimately modulates feeding behaviors. Electrophysiological studies of individual taste cells in the tongue show that a majority of taste cells respond to more than one of the four classic tastes, and many respond to three or four of these tastes. Similarly, primary afferent taste axons also show a wide variety of response profiles, as do taste-response neurons within brainstem relay nuclei. Taken together, these findings argue against a strict "labeled line" theory in which individual tastes would be passed through separate communication channels. Rather, taste information appears to be encoded in a more complex "across-fiber" pattern, in which basic tastes are conveyed by a variety of overlapping channels (Saper et al., 2002). Mechanisms by which pleasurable and aversive cues are extracted from such diverse response profiles are unknown but represent an intriguing problem in sensory information coding. Taste information passes through two brainstem relays, the first located in the nucleus of the solitary tract and the second in the parabrachial nucleus. The parabrachial nucleus in turn projects to a gustatory relay nucleus in the thalamus as well as to the lateral frontal cerebral cortex, the central nucleus of the amygdala, and several hypothalamic targets, including the lateral hypothalamic area (Bester et al., 1997). Early electrolytic lesion studies suggested that the gustatory thalamus was critical for hedonic aspects of taste, but more recent studies using smaller, cell-specific lesions showed that after gustatory thalamic lesions, robust taste preferences and taste-conditioned behaviors persist. Hence, subcortical areas are likely to play a critical role in mediating the motivational qualities of taste, but further research is needed to understand these potentially critical interactions.

8.2. Reward System for Feeding

Motivation and reward have been studied most extensively in the context of drug addiction (Wise, 1998) and intracranial self-stimulation (Wise, 2002), where brain reward systems have been identified that allow the reinforcement of responses that have no homeostatic value. However, a number of studies have suggested that food rewards and drug rewards may share

some common neural substrates, including substantial evidence that opioid receptors play key roles in both feeding and reward (Kelley et al., 2002). Recently, Hayward et al (Hayward et al., 2002) reported that mice lacking either enkephalin or β-endorphin peptides showed a deficit in the ability of food reward to increase bar pressing behavior, regardless of the palatability and nutrient content of the foods examined. Interestingly, this deficit was abolished in mice that were fasted prior to the trials. The authors hypothesized that both peptides contribute to the reinforcing nature and hedonic value of food intake. Moreover, as this deficit is not observable following a fasting, homeostatic systems can override the opioid reward pathways during periods of energy demand (Hayward et al., 2002). Put another way, endogenous opioid systems regulate the hedonic value of food intake independently from the ongoing metabolic needs of the individual. Furthermore, food deprivation, which enhances the hedonic response to food, also increases the motivational value of non-food rewards, such as psychostimulants (Cabeza de Vaca and Carr, 1998), intracranial self-stimulation (Carr, 1996), and heroin intake.

The NAc and its dopaminergic inputs have been strongly implicated in drug addiction and intracranial self-stimulation, but early evidence did not show a similar involvement of the NAc in food reward. Rats in which the nucleus accumbens was ablated did not show obvious reductions in food consumption (Ikemoto and Panksepp, 1996), and such animals still showed robust operant responding for food, leading to some speculation that food rewards were not dopamine dependent. However, more recent evidence has again argued for a dopaminergic contribution to food reward. Mice that genetically lack the ability to produce dopamine normally die of starvation. However, they resume feeding after introduction of the tyrosine hydroxylase gene into the caudate-putamen, although introduction of this gene into the NAc alone is insufficient to restore feeding (Saper et al., 2002).

Pharmacological studies have also shown that the NAc plays an important role in regulating feeding behaviors, as feeding was potently induced by injections of opioid agonists, GABA agonists, glutamate antagonists, or nociceptin into the nucleus accumbens (Stratford and Kelley, 1997). These injections preferentially increased consumption of palatable foods, such as sweet, salty, or fatty foods or liquids, over consumption of water or less palatable foods, suggesting that increased food intake did not merely reflect generalized increases in motor activity. Mu and kappa opioid antagonist injections into the nucleus accumbens inhibited feeding and sucrose intake, again with preferential reductions in the consumption of sucrose versus less palatable substances. Thus, NAc circuits may play an important modulatory role, particularly in promoting the intake of food that is hedonically desirable. The mechanisms of

NAc influences on feeding are not well understood. The NAc has extensive reciprocal interactions with the LHA, which have been hypothesized to regulate feeding behavior (Stratford and Kelley, 1999).

In addition, the main output target of the GABAergic neurons in the NAc is the ventral pallidum, which targets the LHA with its GABAergic neurons(Groenewegen et al., 1993). Thus, the NAc can disinhibit the LHA neurons, much as the striato-pallidal circuit disinhibits motor responses. Feeding induced by injection of GABA antagonists into the NAc can be blocked by GABA antagonists in the ventral pallidum as well as by GABA agonists in the LHA, while conversely, feeding induced by LHA stimulation can be blocked by DA blockade, suggesting that the striatal circuit plays a critical role in expressing feeding behavior. The NAc contains very high levels of MCH receptor mRNA expression and MCH ligand binding sites (Borowsky et al., 2002), suggesting that the MCH neurons in the LHA play a critical role in this relationship. In addition, the NAc may influence feeding behaviors via projections to the motor system, including the substantia nigra pars reticulata, or to the ventral pallidum, which provides a long loop projection to the mediodorsal nucleus of the thalamus (Groenewegen et al., 1993) and then the prefrontal cortex. The latter projection may guide the more complex planning of behaviors that acquire or access foodstuffs.

Although DA and NAc mechanisms are widely believed to be involved in reward processes, it has become increasingly clear that the relationship between DA systems and reward is much more complex than was initially appreciated. The idea that DA is equivalent to hedonic value is most likely oversimplified and has been vigorously challenged and modified (Berridge and Robinson, 1998). Although DA release and DA neuron activity typically increase when rewards are first presented, the DA response eventually habituates even though animals continue to seek the reward long after this habituation occurs (Schultz, 1998b). Furthermore, as the DA response to rewarding stimuli habituates, DA responses become transferred to cues that anticipate and predict future rewards (Schultz, 2002a). Hence, DA function has been proposed to be related more to novelty, attention, or anticipation than to the immediate hedonic impact of the reward itself (Berridge and Robinson, 1998). These emerging views of the DA system may help explain why lesions of DA neurons or the NAc often did not reduce food consumption—in those earlier studies, food was given ad libitum, and obtaining it did not require the instrumental goal-oriented behaviors that are more closely associated with DA function. Non-dopaminergic systems also contribute importantly to the reward value of food, including its immediate hedonic impact. Non-dopaminergic mechanisms of reward have received considerably less study, but serotonin (5HT) has widely recognized influences on

feeding and mood and may have important modulatory effects on reward circuits and motivated behaviors (Fletcher et al., 1999, Pessia et al., 1994). As noted above, serotonin may also directly influence POMC arcuate neurons involved in feeding via 5HT-2C receptors. These POMC neurons may not only release α-MSH but also β-endorphin, which acts at opiate receptors. A prominent ventral striatal role of opiates in food reward has been extensively examined (Kelley et al., 2002), but given the wide distribution of opioidergic neurons and receptors, opioid pathways in other brain areas such as the hypothalamus may also play a role. Finally, endogenous cannabinoids potently influence both appetite and reward in rats and humans (Di et al., 2001), but little is known about their mechanisms of action.

In summary, there is still a long way to go to a clear and comprehensive understanding of feeding-reward interactions. It is not yet clear how hypothalamic pathways that regulate the homeostatic signals for feeding, influence the ability of food to produce pleasure and reward. Interactions between the LHA and NAc, as well as the interactions between DA, 5HT, and opioid systems, are likely to play prominent roles, but the details of these interactions are not well understood. Food rewards are also strongly modulated by taste and satiety cues, but the pathways by which these modulations occur are not well understood. Finally, the basic neural substrates of reward behaviors themselves remain a matter of debate, with a number of competing interpretations of the functions of DA, 5HT, and related circuits. Thus, further inquiry into the need to feed may provide greater insight into the brain systems that underlie normal everyday emotions and motivations.

9. Arousal

As described above, the ability for adequate regulation of feeding is closely correlated with other brain activities. As far as both orexigenic (orexins) and anorectic (CRF) peptides promote arousal it is important to correlate the actions of these peptides with the effects of other substances that are known as arousal-promoting. One such neuropeptide is substance P.

9.1. Substance P (SP)

Substance P (SP) was the first identified neuropeptide. It belongs to the tachykinin family and is widely distributed and extensively studied in the CNS. Injection of SP induces a dopamine-mediated behavioral activation, increasing spontaneous motor and investigatory behavior. In vivo release studies have suggested that SP exerts a tonic excitatory influence on nigrostriatal neurons. SP may activate mesocorticolimbic DA neurons in response to stress (Cador et al.,

1986). A number of studies employed a variety of behavioral paradigms to study the implication of SP in processes of memory and reinforcement. Facilitation of learning by post-trial injection of SP has been observed at injection sites where the peptide has also been shown to be positively reinforcing – in the lateral hypothalamus, medial septum and ventral pallidum. Systemic injection of SP even counteracts age-related learning deficits. SP is released in response to aversive stimulation and modulates defensive reactions after injection into mesencephalic regions. Antidepressant and anxiolytic drugs can reduce SP levels in brain; selective NK1 receptor antagonists produce antidepressant-like effects, while SP agonists had anxiogenic effects in elevated-plus maze (Hasenohrl et al., 2000). In rats, which had been trained to bar press for food reward on a fixed-ratio (FR) 20 schedule, the i.p. injection of SP resulted in a dose-related decrement in response rates. SP (250-500 micrograms/kg) decreased operant responding. The IP injection of morphine (10 mg/kg) markedly suppressed operant responding. So, systemically administered neurokinin SP can affect operant responding for food reward (Hasenohrl et al., 2000). On the other hand, Cador et al showed that SP injection into VTA has no effect on operant (fixed interval) responding behavior. The study of effects of intra-VTA administration of SP on feeding behavior revealed no differences between experimental and saline groups either for eating duration or for latency to eat and amount of food intake, while in a food-deprived condition administration of SP increased latency to eat but not other parameters recorded: food and water intake, time spent drinking and eating. (Cador et al., 1986)

9.2. Histamine (HA)

The neurotransmitter histamine (HA) is involved in many central nervous system functions (Brown et al., 2001b, Haas and Panula, 2003). A number of biochemical, electrophysiological and lesion studies indicated the presence of a neuronal histaminergic system (Haas et al., 1973, Haas and Wolf, 1977); later localization and projection patterns of histamine neurons were established (Panula et al., 1984, Watanabe et al., 1984). These studies showed that histamine neurons are confined to a small region of the posterior hypothalamus — the tuberomammillary nucleus — and that these neurons have widespread, diffuse projection patterns. The highest density of histaminergic fibres is found in the hypothalamus, with all nuclei receiving a strong or moderate innervation. Histaminergic neurons are mainly large cells, approximately 25–30 μm in diameter (Watanabe et al., 1984), which contain, in addition to histamine, a number of other neuroactive substances (or at least their synthesizing

enzymes), including GABA, adenosine, met-enkephalin, galanin, and substance P (Köhler et al., 1985, Ericson et al., 1991). They lie on the ventral surface of the brain and send out several primary dendrites, which subdivide into long, secondary dendrites. The dendrites from a single neuron often overlap with dendrites from other histamine neurons. Some of these dendrites project to the mamillary recess or the surface of the median eminence and come into contact with the CSF (Ericson et al., 1987). Substances present in the CSF, may, thus, influence the firing of histaminergic neurons.

9.2.1. HA promotes arousal

Numerous studies confirm the involvement of the histamine system in arousal mechanisms. Histamine neuron firing varies across the sleep-wake cycle and a circadian rhythm of histamine release has been demonstrated, with higher HA release in periods with high locomotor activity. Icv injections of HA cause phase-shifts in locomotor activity; in vitro, HA changes the firing rate of suprachiasmatic nucleus neurons (Cote and Harrington, 1993). Injection of histidine decarboxylase inhibitor reduces light-induced circadian phase-shifts and disrupts free-running circadian rhythms of locomotor activity. Bilateral transection of the posterior hypothalamus, at the level of the tuberomamilary nucleus, or injections of the GABA-A agonist muscimol to this region lead to somnolence or hypersomnia (Lin et al., 1989). H_1 receptor antagonists, the classical antihistaminics, cause sleepiness. Upregulation of neuronal histamine levels by oral administration of histidine or by the H_3 receptor antagonist, thioperamide, strongly enhance wakefulness. Histaminergic fibres innervate cholinergic neurons in the basal forebrain which provide input to the cortex, and those in the pedunculoponine nucleus of the brain stem which provide input to the thalamus; both nuclei promote arousal and in vitro HA depolarizes both groups of neurons (Brown et al., 2001b).

9.2.2. Action of HA on food intake

Increase in central histamine produced by icv injection of HA, L-histidine, or application of thioperamide supresses feeding, whereas H1-R antagonists increase food intake (Ookuma et al., 1993). Depletion of neuronal histamine enhances feeding-associated locomotor behaviour in the phase of circadian cycle when histamine release is normally high. Zucker (obese) rats have a lower hypothalamic HA level and are not responsive to perturbations of the HA system. The H_1 receptor antagonists, chlorpheniramine and mepyramine, were found to induce feeding and to suppress glucose-sensitive neurons located in the ventromedial

hypothalamic nucleus but not those in the paraventricular nucleus or lateral hypothalamus (Fukagawa et al., 1989).

9.2.3. HA is suggested to inhibit reinforcement

H1 receptor antagonists can act as reinforcers, either when applied alone or in combination with other reinforcers such as opiates, cocaine and amphetamine. The following facts suggest that histamine itself is a reinforcement inhibitor: injection of HA into the lateral ventricle caused an increase in reinforcement threshold and injection of histamine into the lateral hypothalamus specifically suppress self-stimulation (Cohn et al., 1973); Loading with L-histidine, which leads to enhanced histaminergic tone, inhibited the morphine-induced place preference (Suzuki et al., 1995). Several studies of the effects of tuberomamillary nucleus (TM) lesions demonstrated a facilitation of self-stimulation (Wagner et al., 1993) and of the performance of rats in a variety of learning tasks, including a habituation paradigm, aversively motivated learning tasks and water maze (Huston et al., 1997), suggesting a functional antagonism between histaminergic and dopaminergic systems that is quite surprising taking into account the histamine-mediated arousal and the fact that other drugs that induce wakefulness and arousal usually increase dopaminergic tone.

9.3. Modafinil.

There are a number of uncertainties about the molecular bases of other efficacious wake-promoting compounds - amphetamines and modafinil. Amphetamines block plasma membrane transporters for DA, NE, and 5-HT and inhibit the vesicular monoamine transporter (VMAT2), releasing monoamines from the synaptic vesicles into which VMAT2 pumps them (Seiden et al., 1993). Noradrenergic mechanisms have been proposed to explain the wake-promoting effects of amphetamine-like stimulants. However, dopamine-specific reuptake blockers can promote wakefulness in normal and narcoleptic animals better than NE transporter-selective blockers (Nishino and Mignot, 1997). Furthermore, the wake-promoting effect of amphetamine is maintained after severe reduction of brain norepinephrine produced by lesions of the noradrenergic cells of the locus coeruleus in cats (Jones et al., 1977).

The mode of action of modafinil, a new wake-promoting compound (chemically a benzydrylsulfinyacetamid) used in the treatment of sleepiness associated with narcolepsy, is even more uncertain. Studies have suggested that modafinil increases wakefulness by

activating α-1 noradrenergic transmission (Duteil et al., 1990) or hypothalamic cells that contain orexins (Chemelli et al., 1999), or that it may work by modulating GABAergic tone (Ferraro et al., 1996). Central pharmacological blockade or genetic ablation of alpha-1B-adrenoreceptors markedly attenuates the behavioral modafinil-induced activation (Stone et al., 2002). Modafinil exhibits a weak affinity for the dopamine transporter (DAT) (Mignot et al., 1994). To identify the molecular basis for the wake-promoting effects of amphetamines and modafinil, the responses to these compounds were studied in DAT knock-out mice. It was found that the wake-promoting effects of classical stimulants and modafinil are abolished in DAT knock-out mice (Wisor et al., 2001). On the other hand, modafinil, in contrast to amphetamine, was unable to modify in vivo the firing patterns of dopaminergic neurons in substantia nigra and ventral tegmental area and noradrenergic neurons in locus coeruleus (Akaoka et al., 1991). Injections of haloperidol blocked significantly the amphetamine- but not modafinil-induced arousal (Lin et al., 1992). The effects of modafinil in vitro have not been investigated.

10. Background and aims of the study.

Dopaminergic and GABAergic neurons in the ventral tegmental area (VTA) are crucially involved in mechanisms of reward and emotional arousal (Wise and Rompre, 1989). The hypothalamus regulates homeostatic drive to eat and sends a massive output to the VTA, including projections from neurons containing orexins (Fadel and Deutch, 2002). VTA neurons are likely to be involved in both the physiological and pathophysiological roles of orexins. In narcoleptic canines, the presentation of food, one of the stimuli, which potently activate VTA dopamine neurons, is an extremely effective trigger for cataplexy (Nishino and Mignot, 1997). *We hypothesized that orexins could activate midbrain dopaminergic neurons*. However, Okura and colleagues have recently shown that manipulations of dopamine D_2-like (D_2, D_3, D_4) receptors can reduce or enhance cataplexy while leaving REM sleep unaffected (Okura et al., 2000). Dopamine D_2-like receptors in the nigrostriatal system are well known to be involved in the regulation of muscle tone. Thus, patients suffering from Parkinson`s disease exhibit *catalepsy* - an inability to initiate movement, accompanied by enhanced muscle tone. Loss of dopamine neurons in Parkinson`s disease or application of neuroleptics, such as haloperidol (D_2-like receptor antagonist) causes catalepsy via increased firing of GABAergic substantia nigra pars reticulata neurons which inhibit the thalamocortical motor system and medial pontine reticular formation (Niijima and Yoshida, 1982, Boulay et al., 2000; Evans et al., 2001; Blandini et al., 2000). *Thus, our next hypothesis claimed that orexins could affect the GABAergic neurons in SN to maintain the normal regulation of muscle tone, which is missing in narcoleptics.* **Taken together, the first aim of my study was to investigate the action of orexins on dopaminergic and GABAergic neurons in SN and VTA.**

The exact signal transduction mechanism of orexins' effects is not yet known. Orexin receptors are coupled to phospholipase C (Sakurai et al., 1998;van den Pol et al., 1998). In TM neurons orexin activates the Na+/Ca2+ exchanger (Eriksson et al., 2001). In dorsal raphe the expression of orexin receptors is correlated with the expression of TRP6 and TRP5 subunits of transient receptor potential (TRP) channels (Sergeeva et al., 2003). These data suggest a specific mediation of orexin effects in different neuron types. As orexins increase intracellular calcium in acutely isolated A10 dopamine neurons (Nakamura et al., 2000), *we hypothesized that the action of orexins is dependent on the intracellular or extracellular concentration of* Ca^{2+}. *We also hypothesized that the mechanism of orexins' action could be*

similar in all aminergic nuclei. **The second aim was to study the mechanisms of orexin-mediated excitation in SN and VTA.**

Several recent studies showed a number of differences in the electrophysiological characteristics of DAergic neurons in the VTA according to the presence or absence of calcium-binding protein, calbindin (Liss et al., 2001; Neuhoff et al., 2002). As we found that orexin affected subgroups of DAergic neurons in VTA in different ways, *we hypothesized that these subgroups could vary in their electrophysiological characterisctics such as firing frequency, spike threshold, AHP amplitude, etc. The other possibility was that expression of orexin receptors could be related with expression of other food-related peptides such as neuropeptide Y, or their receptors.* Therefore, t**he third aim was to investigate the differences in electrophysiology and expression patterns of these groups.**

A10 dopamine neurons project from the VTA to the nucleus accumbens (NAcc), amygdala, hippocampus and prefrontal cortex, forming the mesocorticolimbic dopamine system (Albanese and Minciacchi, 1983). NAcc is also implicated in reward-related processes. Stimulation of NAcc shell elicits feeding. (Maldonado-Irizarry et al., 1995). Furthermore, it is known that neurons in the NAcc shell project directly to the LH (Kirouac and Ganguly, 1995), so they could have synapses on orexins neurons which are exculsively expressed there. *Thus, we found that it is important to study whether orexins affect NAcc directly*. **The fourth aim was an analysis of orexin effects on the nucleus accumbens.**

A number of further hypothalamic peptides modulate food intake and arousal in rats. The exact mechanisms by which these lateral hypothalamic neurons drive feeding have not been worked out. Several studies pointed out that these peptides could affect the dopaminergic system: alpha-MSH administered into the VTA induced a significant increase in dopamine and DOPAC levels in the nucleus accumbens (Lindblom et al., 2001). Intra-VTA injection of CART induced locomotor activity and promoted conditioned place preference in rats (Kimmel et al., 2000). Another interesting question is the interaction of orexigenic and anorectic peptides on the same neurons. It has been shown that corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) exert opposite effects on eating behavior in the hypothalamus (Heinrichs et al., 1993) and on anxiety in the amygdala cells (Sheriff et al., 2001). *Our initial hypothesis suggested that neuropeptides that increase food intake, would excite DAergic VTA neurons, whereas anorectic neuropeptides would inhibit DAergic VTA neurons.* **The fifth aim was to investigate the possible mediation of neuropeptide interactions by the dopaminergic system.**

Stronger arousal-promoters than the neuropeptides are the neurotransmitter histamine and a novel wake-promoting compound modafinil. Histamine (HA) is involved in many central nervous system functions (Brown et al., 2001b). Data concerning the effect of HA on the dopamine system are contradictory. On the one hand, it was shown that i.p. administration of H_1 antagonists produced increases in both neostriatal and accumbens levels of DA in anaesthetized rats (Dringenberg et al., 1998; Masukawa et al., 1993), on the other hand, according to Fleckenstein et al., (Fleckenstein et al., 1993), i.c.v. administration of histamine increased DOPA accumulation and DOPAC concentration in the nucleus accumbens (Nacc), but not in the striatum, suggesting the stimulation of mesolimbic, but not nigrostriatal, DA neurons, through an action at the H_1 receptor. Application of histamine in the NAcc of anesthetized rats enhanced extracellular dopamine levels in a dose-dependent way, which was also H_1-dependent (Galosi et al., 2001). A functional antagonism between histaminergic and dopaminergic systems has been put forward by (Huston et al., 1997). The cellular basis for such an antagonism is unknown. *As these contradictory facts could not lead to a straight conclusion on this important issue, we performed an in vitro study to investigate the effect of histamine on SN and the VTA.* **The sixth aim was to examine the effect of bath-applied histamine on the neurons in SN and VTA**.

The exact mechanism of action of modafinil is not known. Some authors insist that its effects are mediated by the noradrenergic but not the dopaminergic system (Lin et al., 1992) and claim that modafinil has different effects than amphetamines that have side effects such as the development of tolerance and physical dependence. Some evidence supports the hypothesis that the actions of modafinil are linked to the dopaminergic system: modafinil exhibits a weak affinity for the dopamine transporter (DAT) (Mignot et al., 1994) and wake-promoting effects of classical stimulants and modafinil are abolished in DAT knock-out mice (Wisor et al., 2001). *We suggested that modafinil could produce the effect that is similar to amphetamine, i.e., inhibition of DAergic neurons in SN and VTA.* No study about effects of modafinil in vitro has been done yet. **The seventh aim was to investigate the effect of modafinil on the neurons in VTA in vitro**.

11. Methods

11.1. Electrophysiological recordings

11.1.1.Solutions

11.1.1.1. Recording solution

Extracellular and intracellular recordings in brain slices were performed in artificial cerebrospinal fluid (ACSF), which contained (in mM) 124 NaCl, 3.7 KCl, 25.6 $NaHCO_3$, 1.24 NaH_2PO_4 , 2 $CaCl_2$, 1,3 $MgSO_4$, and 10 glucose, adjusted to pH 7.4, and possessed an osmolarity of ~300 mOsm. It was constantly bubbled with carbogen (95% O_2/5% CO_2). The ACSF was made fresh daily by adding the appropriate amount of glucose and bicarbonate. During preparation of stock solutions, $CaCl_2$ was first dissolved in a clean glass and then added last to the stock solution, after the complete dissolving of all other components to prevent precipitation of calcium carbonate. In one series of experiments low (0.2 mM) Ca^{2+} /high (4.0 mM) Mg^{2+} solution was used (the concentration of all other components was the same as in described above ACSF).

11.1.1.2. Cutting solution

For the slice preparation a neuroprotective cutting solution was used, where NaCl is replaced with an equimolar concentration (207 mM) of sucrose (Aghajanian and Rasmussen, 1989). This replacement reduces sodium influx, which occurs with the tendency of neurons to depolarize during the anoxia that accompanies decapitation, dissection and slicing; and it decreases the neurotoxic effects (excitotoxic cell swelling) from passive chloride influx followed by cation and water entry. The concentration of all other components was identical to ACSF that was used during recordings. The indicator Phenol red (phenolsulfonphtalein) was added to this solution to monitor the pH. Before slice preparation, a necessary amount of the cutting solution (about 200ml) was saturated with 95% O_2/5% CO_2 and frozen to obtain an ice-cold solution.

11.1.1.3. Patch pipette solution

Patch pipettes were filled with an intracellular solution containing in mM: potassium gluconate 135, NaCl 5, $MgCl_2$, HEPES 10, EGTA 0.1, Na_2ATP 2, NaGTP 0.5, 0.5 % Biocytin (pH 7.25 with KOH, 280 mOsm). Patch-pipette solutions were aliquoted into 1ml Eppendorf-vials and stored at –30°C. Fresh pipette-solution was thawed and filtered (using syringe filter) before use.

11.1.2. Slice preparation

The brain slices were prepared from 3-4 week old male, Wistar rats. In several experiments 2-week old rats were used, but the data obtained from these animals were always compared with the recordings obtained from the older rats to rule out that immaturity influenced the results. Young animals (3-4 weeks) were used for two reasons. First, young tissue is much more resistant to the trauma of slice preparation than adult tissue. Second, it is much easier to see individual cells clearly in brain slices prepared from young rats, because myelination is not completed in these rats.

All experiments were conducted in compliance with German law and with the approval of the Bezirksregierung Duesseldorf. All efforts were made to minimize the pain and discomfort of the experimental animals. The animals were quickly decapitated with a pair of big scissors; the dorsal end of the skull was cleared of skin and muscles, one branch of a small scissors was inserted in the skull as close as possible to the bone and a longitudinal cut from the dorsal end of skull to the line between the eyes was made. To ease the following removal of the brain from the skull, additional cuts were made to the left and the right sides of the initial place of scissors insertion (the dorsal end of skull) and between the eyes (**Fig.1A**). These cuts left two plates of skull covering each hemisphere. A small pair of pincers was used to grasp each part of the skull and remove it. Dura mater was also carefully removed with the pincers. After the brain was fully exposed, a spatula was quickly inserted along the front portion of the brain and was slid forward to cut the olfactory bulbs. Afterwards the brain was completely pushed out of the skull cavity with the help of a spatula and put into a glass with ice-cold, oxygenated sucrose-ACSF. It was chilled there about 2-3 minutes; then a brain block containing the VTA or striatum was cut with a scalpel and glued with Permabond-plastic bonding adhesive to the platform of a slicer chamber (**Fig.1B**).

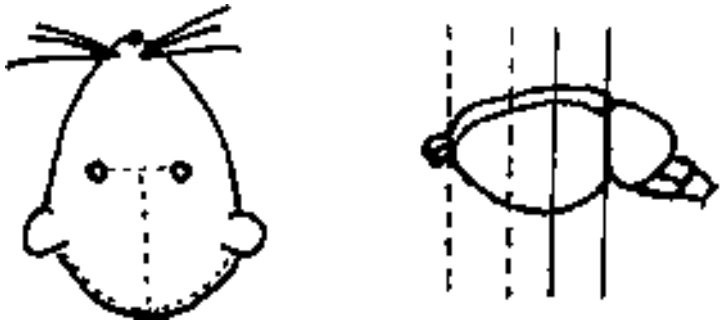


Fig.1. **A.** The removal of a brain from the skull. **B.** A brain block that contains straitum (dotted lines) or the VTA (continuous lines).

400 μm thick coronal slices were cut at the level of the rostral VTA (-5.4 to –6.0 mm from Bregma according to the atlas of Kruger et al., 1995, or striatum (1.5 to 0.9 mm from Bregma according to the same atlas) using the vibroslicer (Campden Instruments, U.K). The slices were placed into the chamber filled with constantly bubbled ACSF (containing NaCl) for at least 1 hour at room temperature. The slices rested there upon a nylon mesh which permitted exposure of ACSF from both surfaces of the slice. After 1 hour the slice was transferred to the recording chamber and secured by placing a net atop the slice. There they were constantly perfused with the same ACSF at a flow rate of 1 ml/min. During flowing in a plastic tube from the upper vessel to the slice, the ACSF was heated to 32°C by a heating system containing a thermostat and a heater.

11.1.3. Extracellular single-unit recordings.

Extracellular recordings were obtained using pipettes made from borosilicate glass tubes and filled with 2M NaCl immediately before use. The resistance of these microelectrodes was 3 - 10 MΩ. An Ag/AgCl wire that conducts the signal to the headstage of the amplifier Axoclamp 2A or 2B (Axon Instruments, USA), was inserted in the glass microelectrode and the electrode was tightly fixed on the headstage. The reference Ag/AgCl wire was fixed in chamber and connected to the headstage as well. Before the experiments, proper grounding of all instruments through low-resistance ground cables was made. An important aspect of reducing noise was to minimize ground loops as well as magnetic and electric line-frequency noise from external sources (computer, monitor, light source) and 50 Hz power lines.

Positioning of the microelectrode was controlled under a dissecting microscope using the accessory optic tract as a marker, which is the border between the substantia nigra and VTA. Under visual control the electrode was slowly advanced through the slice with a Micromanipulator 5171 (Eppendorf) until spontaneous activity was detected on the oscilloscope (Hameg). During recording the signals were amplified further by a differential amplifier, digitized by Digidata 1320A(Axon Instruments, USA), filtered between 0.5-10kHz, sampled at 20 kHz and analyzed with pClamp8 software (Axon Instruments, USA). The frequency of extracellular action potentials was determined online in bins of 15 s duration.

11.1.4. Whole-cell patch-clamp recordings

Intracellular recordings were made using the "blind" whole-cell patch-clamp technique (Staley et al., 1992) or IR-DIC for visualising neurons (Moyer and Brown, 2002). Patch pipettes (3-6MΩ) were pulled from borosilicate glass (GB 150F-8P, Science Products, Hofheim, Germany) and filled with intracellular pipette solution. The electrodes were filled only to one-third because the fluid in the back of electrode can cause internal noise-generating films and allow fluid into the holder. In contrast to sharp electrode recordings that utilize pipettes with resistances higher than 50 MΩ, comparatively blunt low-resistance pipettes are used for whole-cell patch clamp recordings. This is done because series resistance should ideally be two orders of magnitude below the cell's resistance, and blunt electrodes (1-2μM) are required to achieve and maintain mechanically stable electrode-membrane seals. To achieve the ideal shape for sealing membrane patches, electrodes were pulled after 5-6 cycles of heating.

The electrode was fixed on the headstage as described above. After attaching the pipette to the headstage, positive pressure is applied by a syringe attached to a plastic tube connected with part of the headstage, the pipette is then lowered to the bath and the amplifier voltage is zeroed. While the microelectrode is advanced toward the slice, its resistance is monitored continuously by applying a small negative test pulse (0,2 nA, 10 ms) to the electrode. Once a contact is made with a cell, the electrode resistance spontaneously increases by10-50%, the positive pressure was now released and a gentle suction to the electrode by mouth quickly resulted in the formation of a gigaseal. At this point, seal quality could be improved by applying a negative holding potential to the pipette. Following the formation of a gigaohm seal, the stimulating pulse was shut down and the cell membrane was ruptured by the brief strong pulse of suction providing a low-resistance access to the cell and establishing the whole-cell configuration. Next, by adjusting the capacitance and series resistance (Rs) compensation and gradually increasing the % compensation, effective Rs compensation was made.

Using IR-DIC for visualizing neurons in brain slices (**Fig.2**) gave a lot of benefits, such as an ability to distinguish between healthy and unhealthy neurons (Moyer and Brown, 2002). In healthy, patchable neurons, the cell membrane appeared smooth and bright, the neurons looked 3-dimensional (while unhealthy neurons usually look two-dimensional), the nucleus was not visible in healthy neurons, they displayed a more complicated geometry, while unhealthy neurons were commonly round. Since on the surface of the slice the majority of neurons was damaged during the cutting, neurons that were situated more than 70μm below

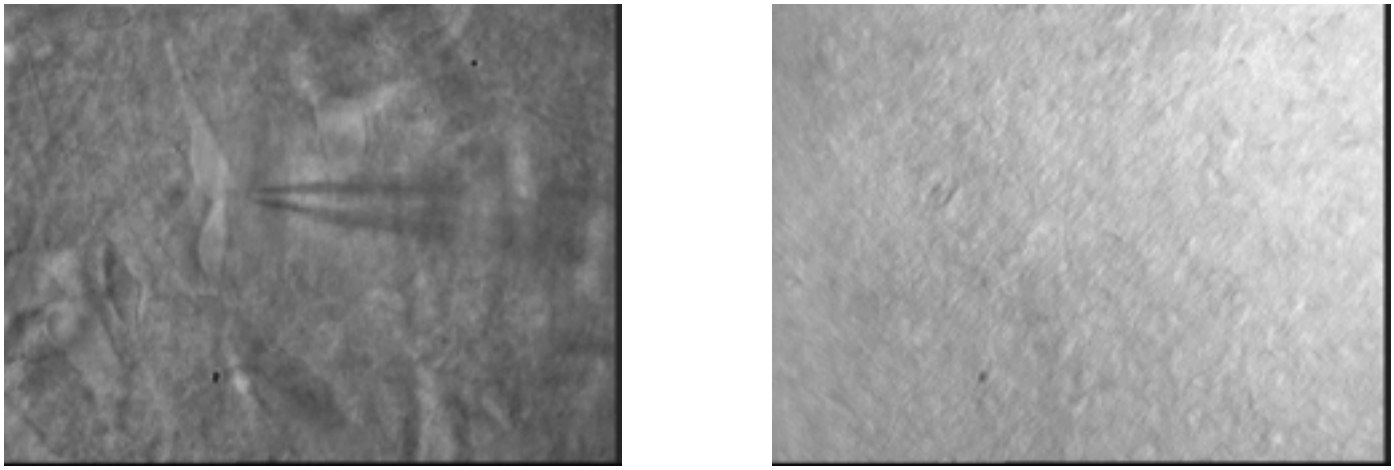


Fig.2. VTA neurons in the slice (40x (left) and 10x (right) magnifications)

the cell surface were recorded. For forming a seal and whole-cell configuration the same steps as in "blind" recordings were used. As the pipette got closer to the cell, the positive pressure could be reapplied if necessary to clear the membrane of debris. The pipette was then lowered onto the cell under visual monitoring until the positive pressure created a small "dimple". At this point the positive pressure was quickly removed (by turning the stopcock) and gentle suction was applied by mouth to obtain a gigaohm seal. The membrane patch was usually voltage-clamped to about-70mV before attempting to go into the whole-cell configuration to eliminate the possibility of a rapid depolarization upon rupture of the membrane. To break in, gentle suction was applied until a sudden increase in the capacitive current was observed as the membrane capacitance of the cell was revealed. Next, the whole-cell capacitance was compensated by adjusting the dial on the amplifier and the series resistance was compensated.

Intracellular signals were recorded using the Axoclamp 2B amplifier in bridge mode. The microelectrode voltages are monitored continuously, and continuous currents can be injected in the cell. Membrane potential measurements were adjusted for a –15 mV liquid junction potential between pipette solution and bath solution. Liquid junction potentials arise from differences in ionic composition and ionic mobilities at the junction between two solutions – the intracellular pipette solution and the extracellular bath solution. Left uncorrected, liquid junction potentials contribute to errors in membrane potential measurements – using K gluconate solution, this error is more than 10 mV. Precise calculation of liquid junction potentials involved the use of the generalized Henderson Liquid junction potential Equation (calculated using pClamp8 Software, Axon Instruments, USA). Associated with the current flow (I) in a microelectrode is a voltage drop across the microelectrode, which depends on the product of the current and the microelectrode resistance (Re). This unwanted IR voltage drop

adds to the recorded potential. The Bridge Balance control is used to balance out this voltage drop so that only membrane potential is recorded. During the repetitive pulses from a stimulator (Master-8), the bridge balance control was advanced from zero until the fast voltage steps seen at the start and end of the current step were just eliminated. The bridge balance was continuously maintained and monitored during current-clamp experiments; the series resistance was < 50 MΩ. Before bridge balancing, capacitance neutralisation was used. The capacitance (C_{in}) at the input of the headstage amplifier is due to the capacitance of the amplifier input itself (C_{in1}) plus the capacitance to ground of the microelectrode and any connecting lead (C_{in2}). C_{in} combined with the microelectrode resistance (Re) acts as a lowpass filter for signals recorded at the tip of the microelectrode. To increase the recording bandwidth, the technique of capacitance neutralization is used that negates C_{in2} and the effective remnant of C_{in1}. The capacitance neutralisation circuit attempts to inject into the headstage input a current which it anticipates to be required to charge and discharge C_{in2} during signal changes. During the repetitive steps the capacitance neutralization control was advanced as far as possible without introducing an overshoot in the step response.

A continuous registration of membrane voltage was done on a Gould TA 550 chart recorder (Gould Electronics, Cleveland, Ohio, USA).

11.1.5. Recording of field potentials in striatum

Coronal striatal slices were prepared as described above (**Fig. 1B**). A bipolar Ni/Cr stimulation electrode was placed within the dorsal or ventral striatum, the nucleus accumbens (NAcc). Constant-current pulses of 80 µs duration (0.3-1.5mA) were applied every 20 s. 15 consecutive responses were averaged off-line to generate one data point. Stimulation strength was adjusted to give a response, which was 50% of the maximum. Glass micropipettes filled with ACSF (3-7MΩ) were placed in the striatum, close to the stimulation electrode, to record extracellular field potentials. Signals were amplified, digitized at 10 kHz and recorded on disc for off-line analysis using pClamp6 software. Field potentials consisted of two negative-going components, referred to as N1 and N2. The N1 component represents the direct activation of medium spiny neuron somata or their axons by the stimulating current, whereas the N2 component is synaptically mediated (Doreulee et al., 2001).

12. Immunocytochemistry

12.1. Immunostaining against orexin A

Deeply anaesthetized 4-5 week-old male Wistar rats were perfused transcardially with 0.9% saline followed by 200 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed in the same fixative overnight at 4°C. A block containing the midbrain was cryoprotected in sucrose, cryosectioned at 34 μm thickness, and the sections mounted on gelatine-coated slides. After drying, the sections were stained according to an immunofluorescence staining protocol. The sections were first preincubated in phosphate buffered saline with 0.25% Triton X-100 and 2% normal donkey serum for 90 min at room temperature. This solution was also used to dilute an affinity-purified goat antiserum against orexin-A (Santa Cruz Biotechnology, Heidelberg, Germany) to 1:500. This antibody-solution was applied to the sections for 12-16 h at 4°C. After washing, the immunoreactivity was revealed by incubation with Alexa Fluor 488-labeled donkey anti-goat IgG (1:500; Molecular Probes, Eugene, OR) for 90 min at room temperature. After coverslipping with phosphate buffered 50% glycerol, the slides were studied and photograped with a fluorescence microscope equipped with the appropriate filters and a digital camera.

In control experiments it was found that preincubation of the orexin-A antiserum with whole orexin-A peptide blocked the immunoreactivity in all studied brain regions. In those experiments we could also exclude artifacts due to binding of secondary antibodies to the tissue.

12.2. Staining against tyrosine hydroxylase (TH) in biocytin-filled neurons.

After recording, slices were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4) for at least 2 weeks, than transfered to 0.1 M phosphate buffer for 24 hours and 30% sucrose in 0,1M PBS for the next 24 hours. Afterwards slices were cryoprotected in sucrose, cryosectioned at 40 μm thickness, and the sections mounted on gelatine-coated slides. After drying, the sections were stained according to the immunofluorescence staining protocol. The sections were first washed in phosphate buffered saline with 0.25% Triton X-100 (PBS-T) for 5 minutes, then preincubated with 2% normal goat serum in PBS-T for 30 min at room temperature. This solution was also used to dilute a mouse monoclonal antibody against tyrosine hydroxylase (TH, Sigma) to 1:500. This antibody solution was applied to the sections for 12-16 h at 4°C. After washing, sections were incubated with Alexa Fluor 488-labeled goat

anti-mouse IgG (1:500; Molecular Probes, Eugene, OR) to reveal the TH immunoreactivity, and Texas Red-streptavidin (1:200; Molecular Probes) to stain biocytin-filled neurons, for 90 min at room temperature. After washing in PBS and coverslipping with phosphate buffered 50% glycerol, the slides were studied and photographed with a fluorescence microscope equipped with the appropriate filters and a digital camera.

13. Single-cell RNA harvest and RT-PCR

For preparation of isolated cells, the ventral tegmental area was dissected from the slice and incubated with papain (Sigma) in crude form (0.3-0.5 mg/ml) for 20 min at 37°C. Thereafter, the tissue was placed in a bath solution with the following composition (in mM): 150 NaCl, 3.7 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, and 10 HEPES, pH 7.4. Cells were separated by gentle pipetting. Neurons visually selected on an inverted microscope were digitally photographed. The electrodes were fabricated from thick-walled borosilicate glass tubes and had resistances of 2-5 MΩ, after filling with solution (in mM: 140 CsCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES/CsOH), sterilizing by autoclave, and adjusting to a pH of 7.2; the neurons were approached with a patch electrode and a gigaohm seal was obtained. After establishing the whole-cell configuration its cytoplasm was sucked into the electrode. This was done under visual control to ensure that only the cytoplasm and not the entire neuron entered the electrode. The content of the electrode was released into an Eppendorf vial with reverse-trancriptase (RT), mixed on a Vortex mixer, centrifuged and incubated at 37^{o}C for 3 hours. After 1 hour 1µl of RT was added to the probe. Afterwards the probes were frozen and stored at –30^{o}C. Cell identification was verified by reverse transcriptase (RT)-PCR (polymerase chain reaction) analysis of tyrosine hydroxylase (TH), GAD 65 and GAD 67 expression.

PCR allows to amplify effectively the DNA from a selected region of the genome. Every cycle doubles the amount of DNA synthesized in the previous cycle, the products of each cycle serve as the DNA templates for the next. Each cycle consists of the following steps – denaturation - separation of the DNA strands, annealing – hybridisation of sense and antisense primers with appropriate DNA strand and extension – DNA synthesis from primers. A two-round amplification strategy was used in each protocol. In each round 35 cycles of the following thermal programs were used: denaturation at 94°C for 48 sec, annealing at 53°C for 1 min, and extension at 72°C for 90 sec. The thin-walled PCR tubes contained a mixture of first-strand cDNA template (2-5 µl), 10× PCR buffer (5 µl), a 200 µM concentration each of deoxyNTP (dNTP), a Mg^{2+} (2-3 mM), a 10pM concentration of each sense and antisense primer, and 2.5 U of *Taq* polymerase. Sense and antisense primers (oligonucleotides) were

designed using BLAST and Primer Design programmes. The following criteria were used: melting temperature – 52-57 degrees, AT/GC – 40-60%, length –about 20 b.p.; possibility of complementary base-pairing between the primers, self-homology and specificity of primers for amplification of the chosen product were also tested. All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). The Taq DNA polymerase is isolated from a thermophilic bacterium, so it is stable at high temperatures and is not denaturated by the repeated heat treatments. We used Platinum Taq DNA Polymerase that is recombinant Taq DNA polymerase complexed with proprietary antibody that inhibits polymerase activity. Due to specific binding of the inhibitor, this Taq polymerase is in inactive form until heat denaturation is performed. It provides an automatic "hot start" that increases sensitivity, specificity and yield of reaction.

The final reaction volume was adjusted to 10 µl with nuclease-free water (Promega, Madison, WI). For the second amplification round, 1 µl of the product from the first amplification was used as a template. The *Taq* enzyme, PCR buffer, Mg^{2+} solution, and four dNTPs were all purchased from Qiagen (Erkrath, Germany). Annealing temperature and Mg concentration were empirically modified to find optimal conditions that result in specific PCR products. Amplifications were performed on a thermal cycler (Mastercycler; Eppendorf).

The protocols of the RT reaction and PCR amplification were similar to those described previously (Vorobjev et al., 2000). Sequencing of the amplification products, which was done on an automatic sequencer (model 377; Applied Biosystems International, Weiterstadt, Germany), revealed the identity of the ampified products (by comparison with known sequences of a given product in the GenBank). The following primers were used (from 5´to 3´): TH (sense): GCTGTCACGTCCCCAAGGTT; antisense: AAGCGCACAAAATACTCCAGG; antisense2 (for the second round): CAGCCCGAGACAAGGAGGAG (size of the product was 220 b.p.); for the PCR analysis of GAD expression in the first round of amplification the degenerated antisense primer: CCCCAAGCAGCATCCACAT was taken either with GAD65 cDNA-specific sense primer - TCTTTTCTCCTGGTGGTGCC or with GAD67 cDNA-specific sense primer TACGGGGTTCGCACAGGTC; for the second round the same sense primers were used in combination with specific antisense primers – CAGTGGTTCCAGCTGTGGC for GAD65 and CGGTTGCATTGACATAAAGGG for GAD67. The following primers were used for the PCR analysis of orexin receptor expression: in the first round of amplification the degenerated primer "dg up" [5'-CTGGC(AT)GATGTGCT(GT)GTGAC-3'] was taken either with OR_1 cDNA-specific lower 1 primer (5'-AACAGCAGAGGGTGGCAGAT-3') or with OR_2 cDNA-

specific lower 1 primer (5'-TGGCTGTGCTCTTGAACATC-3'). For the second round of cDNA amplification, the primers for the OR_1 were upper 2 (5'-TGTTAGTGGACATCACCGAATC-3') and lower 2 (5'-TGAAGCTGAGAGTCAGCACTG-3'); for the OR_2, the lower 2 primer (5'-GGCAATGCAGCTCAATGTAA-3') was used in combination with the degenerated primer dg up. Primers for the 1st amplification of calbindin (CB) and neuropeptide Y (NPY) were described in (Cauli et al., 1997): CB (sense) - AGGCACGAAAGAAGGCTGGAT, CB (antisense) – TCCCACACATTTTGATTCCCTG; NPY (sense) GCCCAGAGCAGAGCACCC, NPY (antisense) – CAAGTTTCATTTCCCATCACCA; for the 2-d amplification the same lower primers were used in combination with designed CB (sense2): TCCTGCTGCTCTTTCGATGC (size of product 303 b.p.) or NPY (sense2): GCTCGTGTGTTTGGGCATTCT (251 b.p.). The following primers for MC4 receptors were used: MC4R (sense) - GAACAATTCTAGTGATCGTGGCG; MC4R (antisense) – GCAGAAGCCTGATTCCACTG, MC4R (antisense for 2d amplification) – GTGCAAGCTGCCCAGATACA; CART (sense)-AGCGAGGAAGTCCAGCAC, CART (antisense)- AAGCATGTGCAAATTACACCC, CART (antisense for the 2d amplification)-CCGAAGGAGGCTGTCACC; substance P (SP, sense) – TGAGCATCTTCTTCAGAGAATCGC, SP (antisense) – ATCGCTGGCAAACTTGTACAACTC, SP (sense for the 2-d amplification) – ATTAATGGGCAAACGGGATGCT.

The following primers were used for simultaneous detection of TRPC channels: In the first round of amplification all 7 cDNAs of TRPC channel subunits were competitively amplified with degenerate primers: Dg up: 5′-TGGGGCC(T/C/A)(T/C)TGCAGA T(A/C)TC(T/A)CTGGGA-3′ and Dg lo: 5′-(G/T)G(A/T)TCG (A/G)GCAAA(C/T)TTCCA (C/T)TC-3′. In the second round 4 subfamilies were amplified separately with the following primers: TRPC1 up: 5′-CAG ATGGGACAGATGTTACAAGA -3′ and Dg lo (expected size of the product 412 b.p.), TRPC2up: 5′-GACATGATCCGGCTCATGTTCAT-3′ and TRPC2 lo: 5′-AGCACAATGACCATGACG-3′(expected size of product 248 b.p.), TRPC4/5 up: 5′-GAATGCT(G/C)C T(G/T)GA(T/C)AT(A/C)CT(A/C)AA-3′ and Dg lo (size of products 389 and 398 b.p.), TRPC3/6/7 up: 5′-GACAT(A/C)TTCAAGTTCATG(G/T)TC(C/A)T -3′and Dg lo (size of products 344 and 345 b.p.). Products of the second amplification: TRPC1, TRPC2 and most of the single cell-derived amplimers of TRPC4/5 and TRPC3/6/7 amplifications were successfully sequenced in the sense direction. Positive controls contained

a mixture of 2 or 3 TRPC isoforms in the case of TRPC4/5 and TRPC3/6/7 subfamilies; therefore they could not be sequenced. In contrast, single neurons expressed as a rule only one member of these two subfamilies. The obtained sequences were found to match known ones (GENBANK accession numbers): rat TRPC1 (AF061266), TRPC2 (AF136401), TRPC3 (AB022331), TRPC4 (AF421368), TRPC5 (AF061876), TRPC6 (AB051213) or high homology with the mouse TRPC7 (AF139923) cDNA. We have submitted the rat TRPC7 cDNA sequence to the GENBANK under accession number AY157999. The identity of amplified TRPC1 cDNA in single cells was verified by digestion of the obtained amplimers with Hha I (223 and 189 b.p. fragments could be seen on the gel).

Competitively amplified cDNAs within each of two subfamilies (TRPC4/5 and TRPC3/6/7) were further digested with the subtype-specific enzymes: MluI (TRPC4-specific, cut TRPC4/5 in two fragments with 145 and 244 b.p. size), BsrSl I (TRPC5-specific, cut TRPC4/5amplimer in two fragments of 52 and 346 b.p. size), Vsp I (TRPC3-specific, TRPC3/6/7 into 61 and 283 b.p.), NcoI (TRPC7-specific, cut TRPC3/6/7 into 98 and 247 b.p.). We failed to find a specific restriction enzyme for the TPRC6 subunit, however MboI proved to be an optimal tool for identification of cDNAs within the TRPC3/6/7 subfamily. It cut TRPC6 into 64 and 281 b.p., TRPC7 into 50, 231, 64 b.p and TRPC3 into 156 and 188 b.p. fragments.

The identity of cDNA sequences was revealed by sequencing of the second round amplification products. Results of amplification were analyzed by agarose gel (1.5%) electrophoresis and staining with ethidium bromide gels. All products of the second round of amplification were purified (PCR purification kit from Qiagen) in water and subjected to sequencing in both directions.

14. Drugs

Drugs used in this study were: Orexin A and B, Melanin concentrating hormone, Neuropeptide Y, Cocaine and amphetamine regulated transcript, Leptin, Corticotropin-releasing factor, alpha-Melanocyte stimulating hormone, Substance P, Agouti-related protein (all from Bachem, Germany), Chelerythrine chloride((Sigma, Germany), DAMGO (Tocris, UK), H7 (Alexis, Switzerland), H89 dihydrochloride (Alexis, Switzerland), Melanin concentrating hormone , Orexin A and B (Bachem, Germany), Quinpirole (RBI, USA), Thapsigargin (Alexis, Switzerland), histamine dihydrochloride (Fluka), mepyramine maleate (Sigma), cimetidine, thioperamide maleate, bicuculline methochloride and CGP55845 (all obtained from Tocris). All other chemicals were obtained from Merck (Germany). Drugs

were stored at room temperature or fridge, dissolved in water (with the exception of CGP55845, thapsigargin, KT5720 and modafinil, dissolved in DMSO. The final concentration of DMSO in the bath was 0.1 %. This concentration of DMSO did not affect the firing rate of cells in control recordings). All peptides were dissolved in 0.9% saline to a concentration of 10 μM and the resulting solution was stored as frozen aliquots at –20 °C. Drugs were bath applied. The aliquots were defrozen immediately before use and diluted in 10ml of ACSF in a medical syringe that was connected by a plastic tube with the recording chamber.

15. Experimental protocols and statistical analysis

Neurons were recorded for at least 15 min to obtain a stable baseline before a bath application of drugs. All recorded neurons displayed a regular firing pattern; burst firing was never observed in control recordings. Only one cell per slice was recorded; one, rarely two slices from one rat were used. The electrophysiological part of the study was made on 348 rats and 389 slices. If two slices from the same rat were used, different experiments (application of different drugs or recording of different cell types – DAergic or GABAergic) were performed. Effects of orexins, histamine and modafinil were studied only in drug-naive slices. During investigation of the signal transduction mechanism of orexin-induced excitation of SN and VTA, as well as during the study of histamine receptor involvement in histamine-induced inhibition of DAergic midbrain neurons, the baseline was recorded for 15 min to be sure that the firing rate of neuron is stable, then one of the inhibitors of signal transduction (for details see section 16.2.5.) was applied and neuronal activity was recorded for the next 15 min to rule out an effect of the inhibitor on firing frequency, and, finally, orexin was applied. This part of the study was performed only with extracellular single-unit recordings to avoid dialysis of the cells that takes place during long-term whole-cell patch-clamp recordings. In the last part of study (section 16.6) two types of experiments were made: effects of several neuropeptides on the same cell were tested by extracellular single-unit recordings: after recording of the baseline for 15 min the first peptide was applied, after the end of the effect (return of the baseline to the initial value) and a stable baseline for the next 15 minutes the second drug was applied and so on. The order of drug application was changed each time to be sure that the previous application did not affect the next one, the action of each neuropeptide in drug-naïve cells was also tested. In the next part of the study whole-cell recordings were used to study the effect of these peptides on the membrane potential of VTA cells.

At the end of each experiment the D_2 receptor agonist quinpirole (10 μM) or dopamine (30 μM) (it does not matter whether to apply quinpirole or dopamine because they both are D_2 receptor agonists) or the μ- receptor agonist Tyr-D-Ala-Gly-NMe-Phe-Gly-ol (DAMGO, 1 μM) were applied to obtain one more evidence for the type of recorded neuron – DAergic neurons in SN and VTA are known to be inhibited by quinpirole or dopamine, but not by DAMGO, whereas GABAergic VTA neurons - by DAMGO, but not by D_2 receptor agonists (Grace et al., 1988; Lacey et al., 1989) The order of application of these substances was chosen during the experiment - these two types of neurons have a number of electrophysiological differences (for details see section 16.1), so one can hypothesize with a high rate of predictability which type of neuron is recorded and apply a substance which is likely to inhibit this cell. If, however, the cell was not inhibited by it in 15 minutes, the inhibitor of the other subtype was applied. Such protocol is routinely used for distinguishing between midbrain DAergic and GABAergic neurons in most in vitro studies(Grace, 1991; Johnson et al., 1992 , Yin et al., 2000, Neuhoff et al., 2002; and others).

The following statistical analysis was performed: the significance of the effect of a given substance (comparison of firing rate or membrane potential of the same cell before and after the application of drug) was tested with Students t-test (paired). All experiments where data from different cells were compared - electrophysiological differences between subtypes of neurons, differences between the effects of orexin A and orexin B, effects of inhibition of signal transduction on orexin-induced excitation, blocking of histamine receptors on histamine-induced inhibition, size of neurons with different molecular profile were studied with Students t-test (unpaired). The relationships between expression of mRNA for neuropeptides, their receptors, calbindin and markers for dopamine and GABA, were investigated with $\chi 2$ test. Data are presented as mean ± standard error of the mean (SEM).

16. Results

16.1. Electrophysiological characterization of the recorded neurons

As described in the introduction, there are two types of neurons in SN and VTA: dopaminergic (DAergic) and nondopaminergic (presumed GABAergic) which can be distinguished according to their electrophysiological and pharmacological properties (Grace and Onn, 1989, Klink et al., 2001).

16.1.1. Properties of DAergic neurons in VTA and SN.

Extracellular single-unit recordings in VTA revealed that presumed dopaminergic neurons had a regular spontaneous activity, fired at lower frequencies in comparison with GABAergic neurons (2.23 ± 0.40 Hz, n=25), had broader action potentials (>2 ms) and were inhibited by the D_2 receptor agonist quinpirole (10 μM) or dopamine (30 μM). Dopaminergic cells in the SN had a firing frequency of 2.3 ± 0.4 Hz and a spike width of 2.63 ± 0.37 ms (n = 9, **Fig.3A, C**) and were also inhibited by quinpirole or dopamine; there were no differences in firing rate and spike width between DAergic neurons in SN and VTA (Korotkova et al., 2002a).

DAergic neurons in the VTA recorded in whole-cell mode possessed the following characteristics: average firing frequency was 2.86 ± 0.32 Hz - burst firing or spontaneous alterations in firing rate were never observed in control cells; spike width was significantly ($p<0.0005$) broader in dopaminergic neurons (3.3 ± 0.13 ms, n=14) than in GABAergic neurons (1.68 ± 0.23 ms, n=7) and spike thresholds were significantly ($p<0.05$) more positive in dopaminergic (-44.7 ± 1.4 mV, n=14) than in GABAergic cells (-50.8±0.7mV, n=7). Dopamine cells possessed a prominent I_h current. I_h sag, measured as the percentage reduction from the peak at the end of a 1 s long step elicited by a –400 pA current injection was 33.1 ± 3.4 % (**Fig.4B**). Input resistance was 266 ± 48 MΩ (n=14). They were inhibited by dopamine or quinpirole. The *posthoc* double stainings also revealed that recordings had been made from TH positive (n=14, **Fig.4A**) cells, which are assumed to be dopaminergic cells (Korotkova et al., 2003).

16.1.2. Properties of GABAergic neurons in VTA and SN.

All recorded nondopaminergic (presumed GABAergic) neurons had a stable spontaneous activity. In SN GABAergic cells had a firing frequency of 8.6 ± 0.8 Hz and a spike width of

1.42 ± 0.17 ms (n = 20, **Fig.3 B,D**). Their firing rate was unaffected by application of quinpirole (10 μM) or dopamine (30 μM). This group in SN was uniform while in VTA the GABAergic cells could be divided into two groups according to their firing rate. One group fired at a relatively high frequency (7.31 ± 1.35 Hz, n=10, **Fig.4C,D**), the second group consisted of slow-firing cells (0.89 ± 0.33 Hz, n = 6, **Fig.4 E,F**). Both groups had the same action potential width (<1.5 ms), were unaffected by quinpirole or dopamine and inhibited by the μ- receptor agonist Tyr-D-Ala-Gly-NMe-Phe-Gly-ol (DAMGO, 1 μM), that provides further evidence for their GABAergic nature (detals were described above, in section 15). Further investigation of properties of these two groups by intracellular recordings in current-clamp whole-cell mode displayed that spike width was significantly more narrow than in DAergic neurons (1.68 ± 0.23 ms, n=7 vs 3.3 ± 0.13 ms, n=14 in DA neurons, $p<0.0005$), spike thresholds were more negative (-50.8 ± 0.7 mV, n=7 vs -44.7 ± 1.4 mV, n=14 in DA neurons, $p<0.05$), they did not possess a prominent I_h current: I_h sag, measured as the percentage reduction from the peak at the end of a 1 s long step elicited by a –300 pA current injection that led to a similar amount of peak hyperpolarization as in DAergic neurons (~ -120mV) amounted only to 5.0 ± 0.2 % vs 33.1 ± 3.4 % in DA cells. Analysis of possible differences between characteristics of GABAergic cells with different firing rates was performed. One group fired at a relatively high frequency: 8.7 ± 2.2 Hz (n=3; **Fig.4D**), that did not differ significantly from frequency of fast-firing GABAergic VTA cells, recorded extracellularly and described above (7.31 ± 1.35 Hz, n=10) . The second group consisted of slow-firing cells: 0.77 ± 0.37 Hz (n=4; **Fig.4F**), which frequency did not differ significantly from slow-firing GABAergic VTA cells, recorded extracellularly and described above (0.89 ± 0.33 Hz, n = 6). Both groups of cells fired at high frequency (>30Hz) during depolarizing current steps; this frequency was not different in the two groups. Cells in both groups were inhibited by the μ- receptor agonist Tyr-D-Ala-Gly-NMe-Phe-Gly-ol (DAMGO, 1 μM) and unaffected by quinpirole (10 μM) or dopamine (30 μM), that provides further evidence for their GABAergic nature (detals were described above, in section 15). The level of DAMGO-induced hyperpolarization did not significantly differ in fast-fired (6.4±1.2mV) and slow-fired (6.1±0.8mV) neurons. These two groups of non-dopamine cells differed in AHP amplitude (10.7 ± 0.9 vs 14 ± 0.9 mV in fast-firing and slow-firing cells, respectively, $p<0.05$), and spike threshold (-52.2 ± 0.4 and 49.4 ± 0.2 mV, respectively, $p<0.005$). Spike width did not differ. Input resistance also did not differ either in dopaminergic (266 ± 48 MΩ, n=14) or GABAergic cells (197 ± 19 MΩ, n=7), or between GABAergic cells with different firing

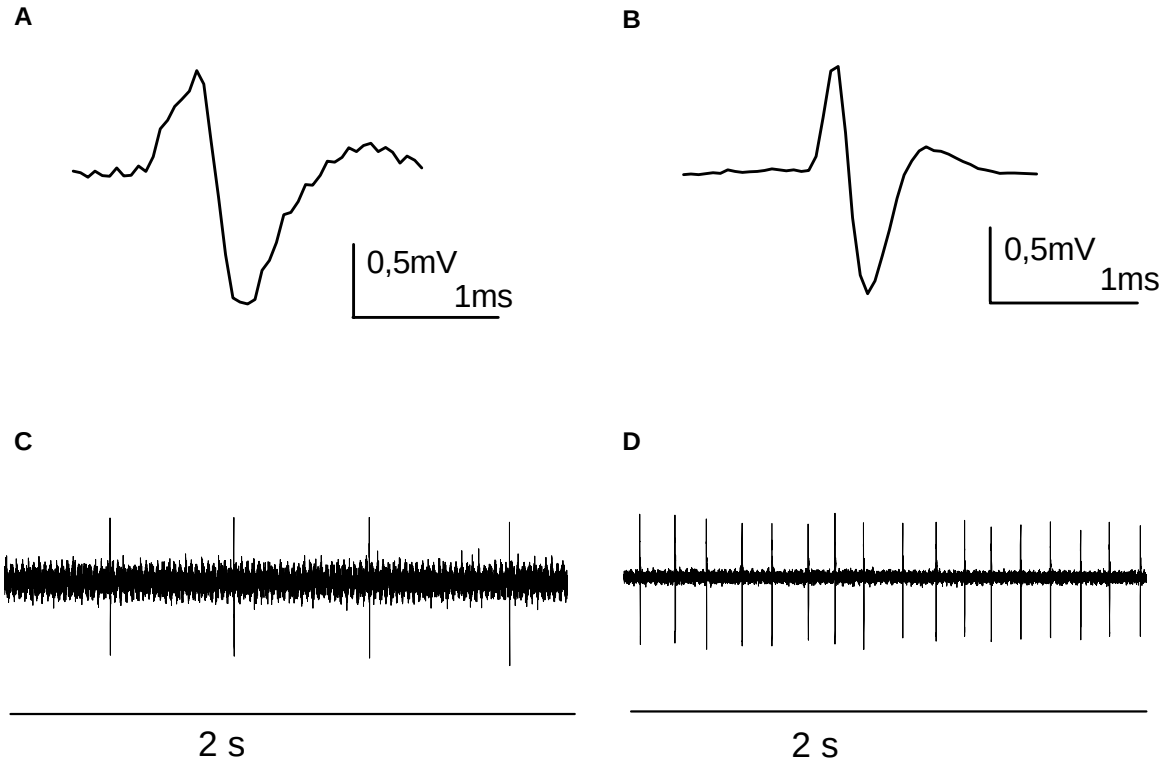


Figure 3. Electrophysiological identification of dopamine and GABAergic neurons in the substantia nigra with extracellular single-unit recording. Dopamine neurons (**A**, **C**) had broader action potentials and slower firing rates whereas presumed GABAergic cells in pars reticulata (**B**, **D**) had narrower action potentials and faster spontaneous firing rates.

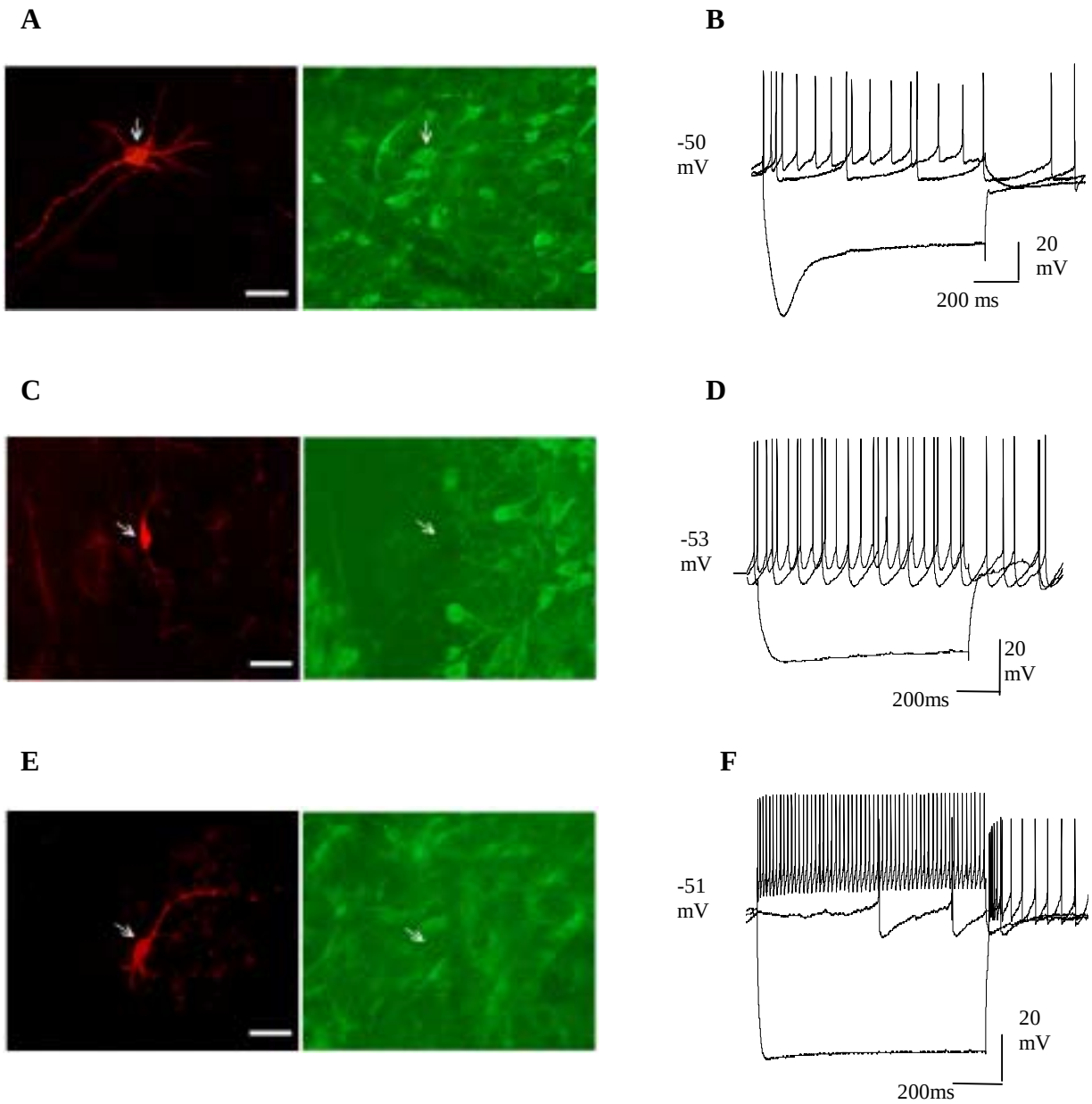


Figure 4. Electrophysiological properties of dopaminergic and GABAergic neurons in the ventral tegmental area.

A. Double stainings of biocytin-filled neuron (red) and tyrosine hydroxylase (TH)-immunoreactive neurons (green). Arrows indicate the position of the neuron in the tissue. This biocytin-filled neuron is TH-positive (scale bar – 50µm). **B.** Voltage responses to current pulses (-0.4pA, 0pA, +0.1pA).

C. Double stainings of biocytin-filled neuron (red) and tyrosine hydroxylase (TH) immunoreactive neurons (green). This neuron is TH-negative. Arrows indicate the position of the neuron. **D.** Voltage responses to current pulses (-0.3pA, 0pA, +0.1pA). This neuron has a relatively fast firing rate.

E. Double stainings of biocytin-filled neuron (red) and tyrosine hydroxylase (TH)-immunoreactive neurons (green). This neuron is TH-negative.

F. Voltage responses to current pulses (-0.2pA, 0pA,+0.1pA). This presumed GABAergic neuron has low spontaneous firing rate.

rates. However, for the detailed investigation of differences between fast-firing and slow-firing GABAergic VTA neurons, additional whole-cell recordings should be performed to increase the number of studied cells in each subgroup.

16.2. Effects of orexins on DAergic and GABAergic neurons in SN and VTA

16.2.1. Responses to orexins in DAergic neurons in SN and VTA.

An overview of this part of the study is presented in **Tables 1** and **2**. Bath application of orexin A (100 nM), which activates both orexin receptors with similar affinity and potency did not affect the spontaneous activity of DAergic neurons in SN pars compacta (n = 16, **Fig.5**) (Korotkova et al., 2002a). This dose was likely to be sufficient to elicit a response - in all published slice experiments with orexins this concentration was sufficient to elicit a response (in responsive cells) and in some cases was sufficient to elicit a maximal effect (Brown et al., 2002); the generation of a concentration-response curve revealed that the EC_{50} for orexin-responsive DAergic neurons in the VTA was 78nM (see below). However, to rule out the possibility of application of a non-sufficient concentration of orexin, we applied higher doses (500 nM, n=4 and 1μM, n=4), but they also had no effect on the firing rate of DAergic neurons in SN.

Most of the substances are known to exert the same effects in SN and VTA, but orexins appeared to be an exception. To characterize the effect of orexins on VTA neurons we performed whole-cell current-clamp recordings in slices (Korotkova et al., 2003). Cells were filled with biocytin by diffusion from the patch pipette and identified post-hoc by staining for tyrosine hydroxylase (TH). To ensure that orexin effects on neuronal firing were not an artifact of dialysis of the cells, the effects of orexins were also investigated using extracellular single-unit recordings.

Three groups of dopaminergic cells in the VTA could be distinguished according to their response to orexin A. In 10 dopaminergic neurons tested extracellularly and 4 dopaminergic neurons recorded intracellularly, there was no effect of orexin A (100 nM) on the firing rate or membrane potential (**Fig.5**). An application of higher dose of orexin (500nM, n=6; 1μM, n=5) was repeated in 40 min in orexin-unresponsive cells. These concentrations also did not influence the firing of orexin-unresponsive cells. To ensure that such repetitive applications will not cause desensitization, we tried this experimental design in orexin-responsive neurons (n=4) and three repetitive applications of the same dose of orexin A (100nM) with an interval of 40-60 min did not elicit significant changes in the level of orexin-induced excitation.

Table 1. Effects of orexins on the firing rate of midbrain DAergic and GABAergic neurons.

Cell subtype	N of experiments	Orexin	Concentration	Effect (increase in firing rate), %
DA SN	16	A	100 nM	No effect
DA SN	4	A	500 nM	No effect
DA SN	4	A	1000 nM	No effect
DA VTA	5	A	10 nM	112 ± 5.%
DA VTA	5	A	50 nM	154 ± 12%
DA VTA	13	A	100 nM	209 ± 24%
DA VTA	4	B	100 nM	205 ± 43%
DA VTA	5	A	500 nM	258 ± 13%
DA VTA	4	A	1000 nM	266 ± 11%
DA VTA	14	A	100 nM	No effect
DA VTA	6	A	500 nM	No effect
DA VTA	5	A	1000 nM	No effect
DA VTA	7	A	100 nM	Oscillatory eff.
DA VTA	9	B	100 nM	Oscillatory eff.
GABA SN	6	B	5 nM	120 ± 8%
GABA SN	12	B	10 nM	150 ± 12%
GABA SN	6	A	100 nM	204 ± 36%
GABA SN	5	B	100 nM	216 ± 23%
GABA SN	4	B	300 nM	220 ± 45%
GABA f.f. VTA	10	A	100 nM	154 ± 21 %
GABA f.f. VTA	5	B	100 nM	149 ± 29 %
GABA s.f. VTA	6	A	100 nM	700 ± 171 %
GABA s.f. VTA	3	B	100 nM	570 ± 113 %

Table 2. Effects of orexins on the membrane potential of DAergic and GABAergic cells.

Cell subtype	N of experiments	Orexin	Concentration	Amount of depo-laritation (mV)
DA VTA	5 (4 under TTX)	A	100 nM	4 ± 0.8 mV
DA VTA	4 (3 under TTX)	B	100 nM	3.8 ± 1 mV
DA VTA	5 (3 with action potential bursts)	B	100 nM	7 ± 0.6 mV
GABA f.f., VTA	3	A	100 nM	3.7 ± 0.3 mV
GABA f.f., VTA	3	B, under TTX	100 nM	3.9 ± 0.4mV
GABA s.f., VTA	4 (2 under TTX)	A	100 nM	6 ± 2 mV

f.f. – fast-firing neurons
s.f. – slow-firing neurons
TTX- tetrodotoxin

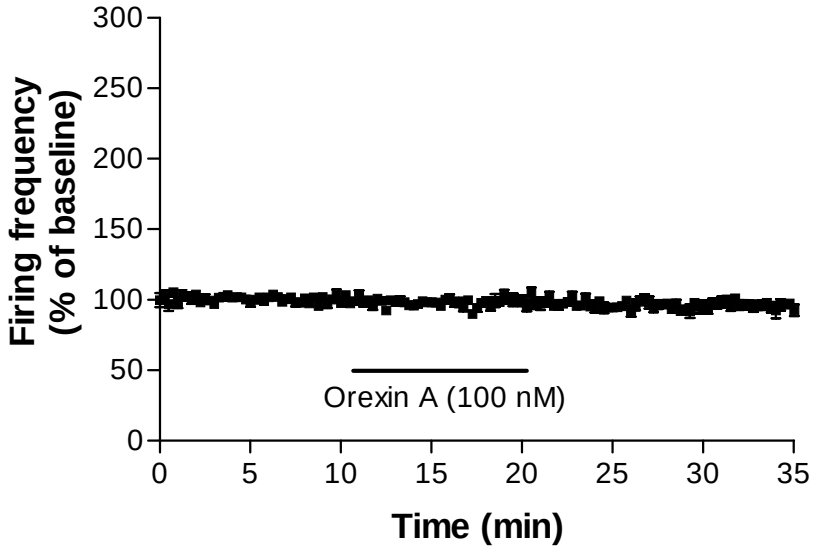


Figure 5. Orexins did not affect the firing rate of a subgroup of DAergic neurons in VTA.

In another group of dopamine neurons, orexin A produced an increase in firing up to 208 ± 35% of baseline rate in extracellular recordings (n=8, $p<0.005$, **Fig. 6A**) – intracellularly, increases in firing were accompanied by a moderate depolarization (4 ± 0.8 mV, n=5, $p<0.005$; **Fig. 6C**) and increase in channel noise. In this group of cells orexin B caused a similar increase in firing rate (205 ± 43%, n=4, $p<0.05$) and amount of depolarization in responsive cells (3.8 ± 1 mV). Two cells recorded extracellularly and 1 recorded intracellularly, which had previously responded to orexin A, did not respond to orexin B. The prior application of the voltage-gated sodium channel blocker tetrodotoxin (0.5 µM) did not prevent the depolarization caused by orexin A (n=4) or orexin B (n=3, **Fig. 6D**). The generation of a concentration-response curve revealed that the EC_{50} for orexin-responsive neurons in the VTA was 78nM (**Fig. 6B)**; the following concentrations of orexin A were tested: 10nM (n=5, non-significant increase in firing rate), 50nM (n=5, $p<0.05$), 100nM (n=10, $p<0.001$), 500 nM (n=5, $p<0.001$) and 1000 nM (n=4, $p<0.01$).

In the last group of dopaminergic cells, after application of orexin A the regular firing pattern was changed to an oscillatory one (**Fig. 7A**) whereby periods of higher frequency firing (1.3–8 Hz, 5-30 s) alternated with silent periods (1-5 s). In extracellular recordings 7 of 25 cells tested responded in this fashion to orexin A (100 nM). Orexin B was also able to elicit this kind of response in extracellular recordings (n=4). Five cells recorded intracellularly demonstrated an oscillatory response to orexin B (100 nM). In three of these five cells strong depolarizations (7 ± 0.6 mV) accompanied by bursts (3-6) of action potentials were interrupted by periods of relative hyperpolarization when the cell did not fire (**Figs. 7B, C**). In one cell silent periods occurred during periods of depolarization (**Fig. 7D**). In the final cell silent periods did not occur but the firing rate changed periodically from a lower rate (0.5 Hz) to a higher rate (4 Hz).

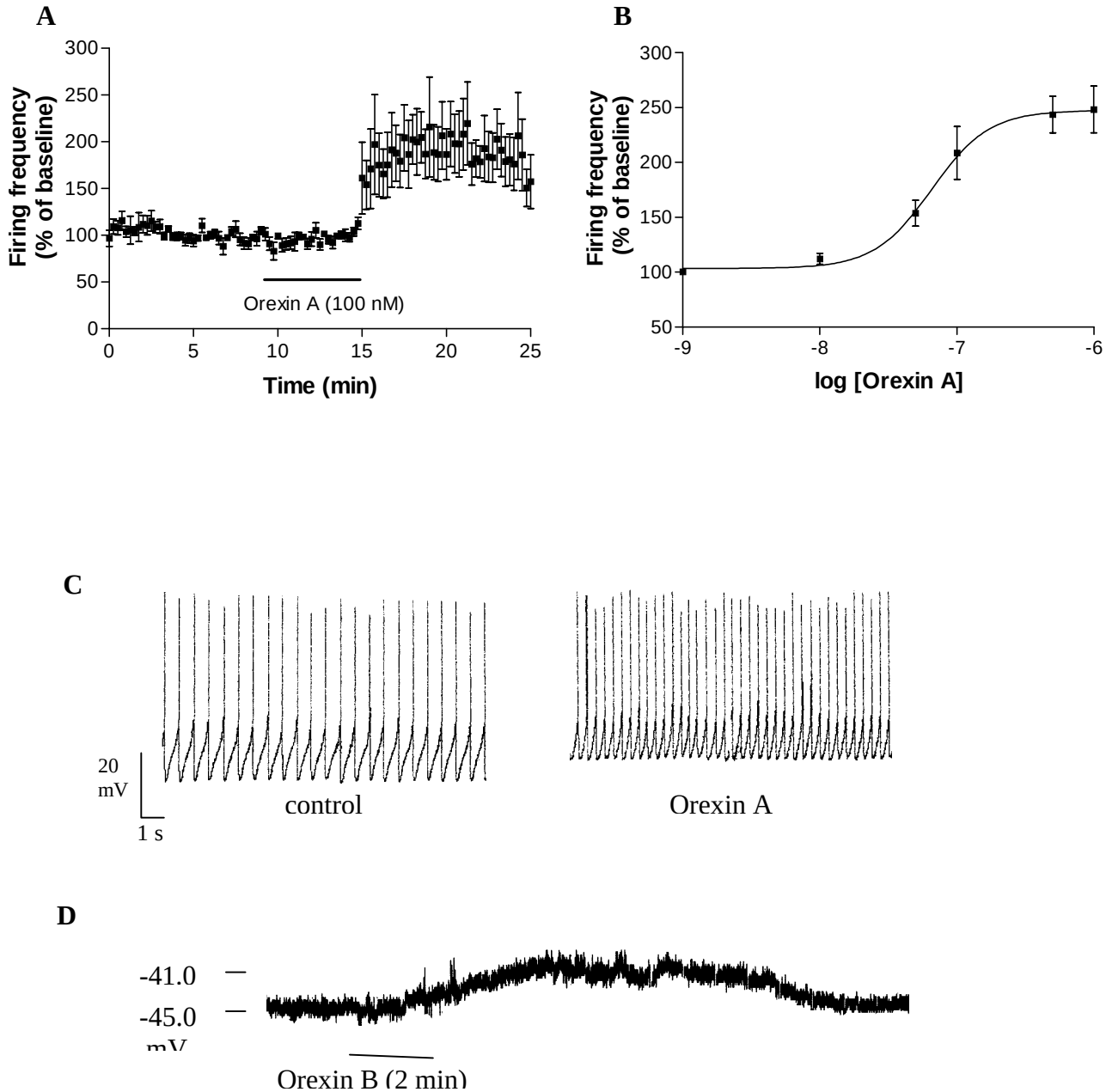


Figure 6. Orexins dose-dependently excited a subgroup of dopaminergic neurons in the ventral tegmental area.

A. Bath-applied orexin A produced an increase in firing up to 208±35 % of baseline rate.

B. Dose-response relationship: EC_{50} for orexin-responsive neurons in the VTA was 78nM. At least 4 neurons were tested at 10, 50, 100, 500 and 1000nM.

C. Chart recording of membrane potential and spontaneous action potentials of the neuron before and after application of orexin B (100nM).

D. Chart recording of the membrane potential of the neuron after application of 0.5µM of TTX. Orexin A causes depolarization of the cell.

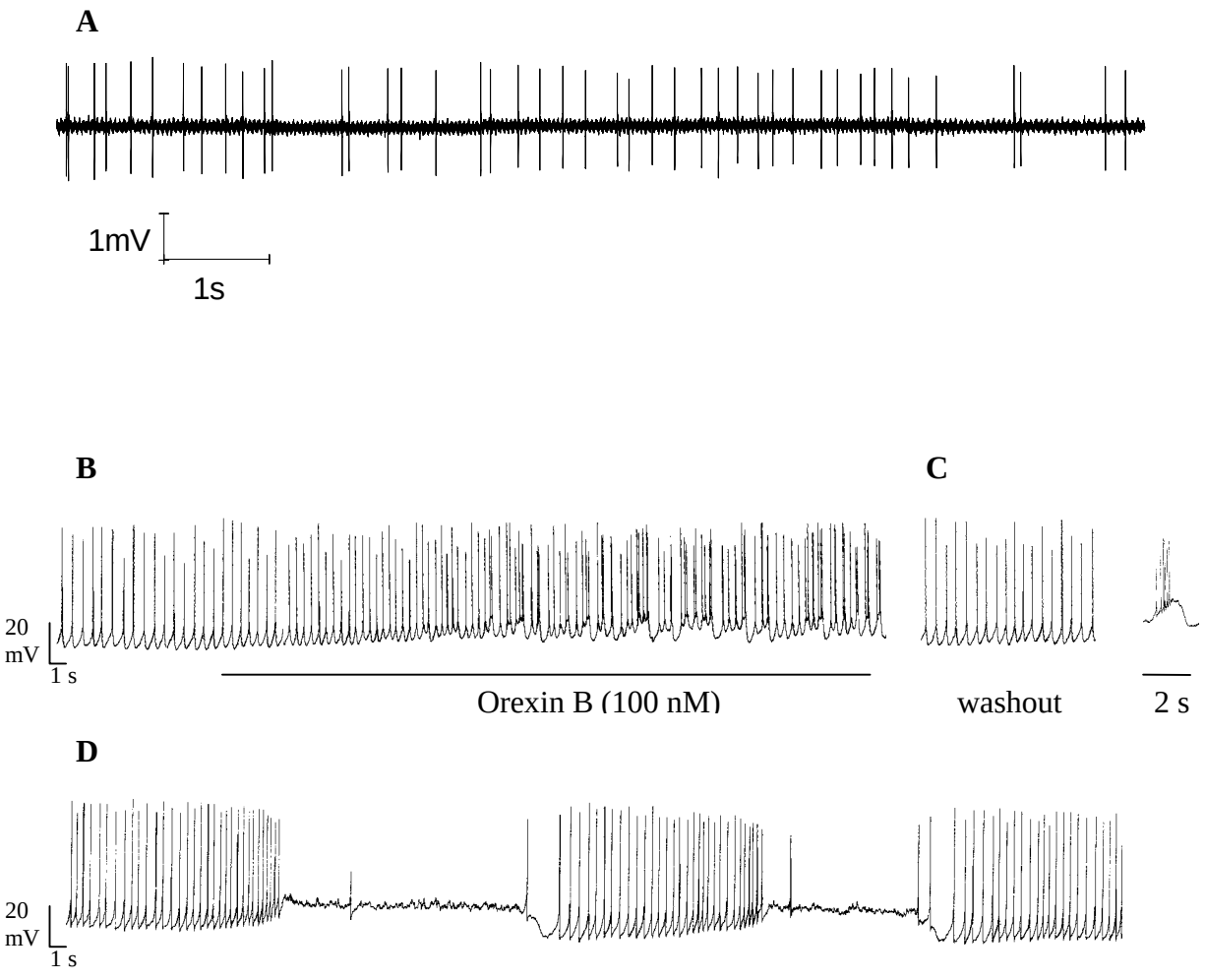


Figure 7.

A. In a subgroup of dopaminergic cells in VTA, after application of orexins the regular firing pattern was changed to an oscillatory one whereby periods of higher frequency firing (1.3–8 Hz, 5-30 s) alternated with silent periods (1-5 s).

B. Example of a TH-positive neuron in which application of orexin B (100nM) caused burst firing.

C. A typical orexin-mediated burst.

D. In one cell silent periods occurred during periods of depolarization.

16.2.2. Responses to orexins in GABAergic cells in SN.

The action of orexins on GABAergic cells appeared to be uniform. In contrast to its lack of effect on DAergic neurons, application of orexin A (100 nM) led to a large increase in the firing frequency of GABAergic neurons in SN pars reticulata to 204 ± 36 % of baseline (n = 6, $p<0.001$ **Fig. 8A**). The application of orexin B (5 - 300 nM), which has a higher affinity for the type II orexin receptor than the type I receptor also led to a large and dose-dependent increase in the firing frequency of GABAergic neurons in SN pars reticulata (n = 27, **Fig. 8B,** for details see **Table 1**). The maximal increase in firing rate elicited by 100 nM orexin A (204 ± 36 %) and orexin B (211 ± 17 %, n = 5, $p<0.005$) were not significantly different. The EC_{50} for orexin B was found to be 12 nM (**Fig. 8C**). None of the GABAergic cells tested were unaffected by the orexins.

16.2.3. Orexin-immunoreactive fibers are present in SN.

This uniform action of orexins on firing rate of GABAergic neurons in SN pars reticulata, was unexpected because a previous study failed to find evidence of orexin fibres in the substantia nigra pars reticulata (Peyron et al., 1998). We re-examined the distribution of orexin-positive fibres in the substantia nigra and ventral tegmental area. Three rats were used for this study. We considered this number of animals to be sufficient because the purpose of this study was to confirm the presence of orexin fibres in SN rather than to give a detailed analysis of them. The most rostral part of the SN reticulata was almost devoid of orexin-immunoreactive fibers, while the density of stained fibers increased caudally (**Fig. 8D**). However, even in the caudal part of the SNr, the fibre density was much lower than in the VTA (**Fig. 8D**). Therefore, we confirmed the presence of orexin fibres in the caudal part of substantia nigra pars reticulata. The detailed quantitative analysis of orexins' immunohistochemistry was not the purpose of this study.

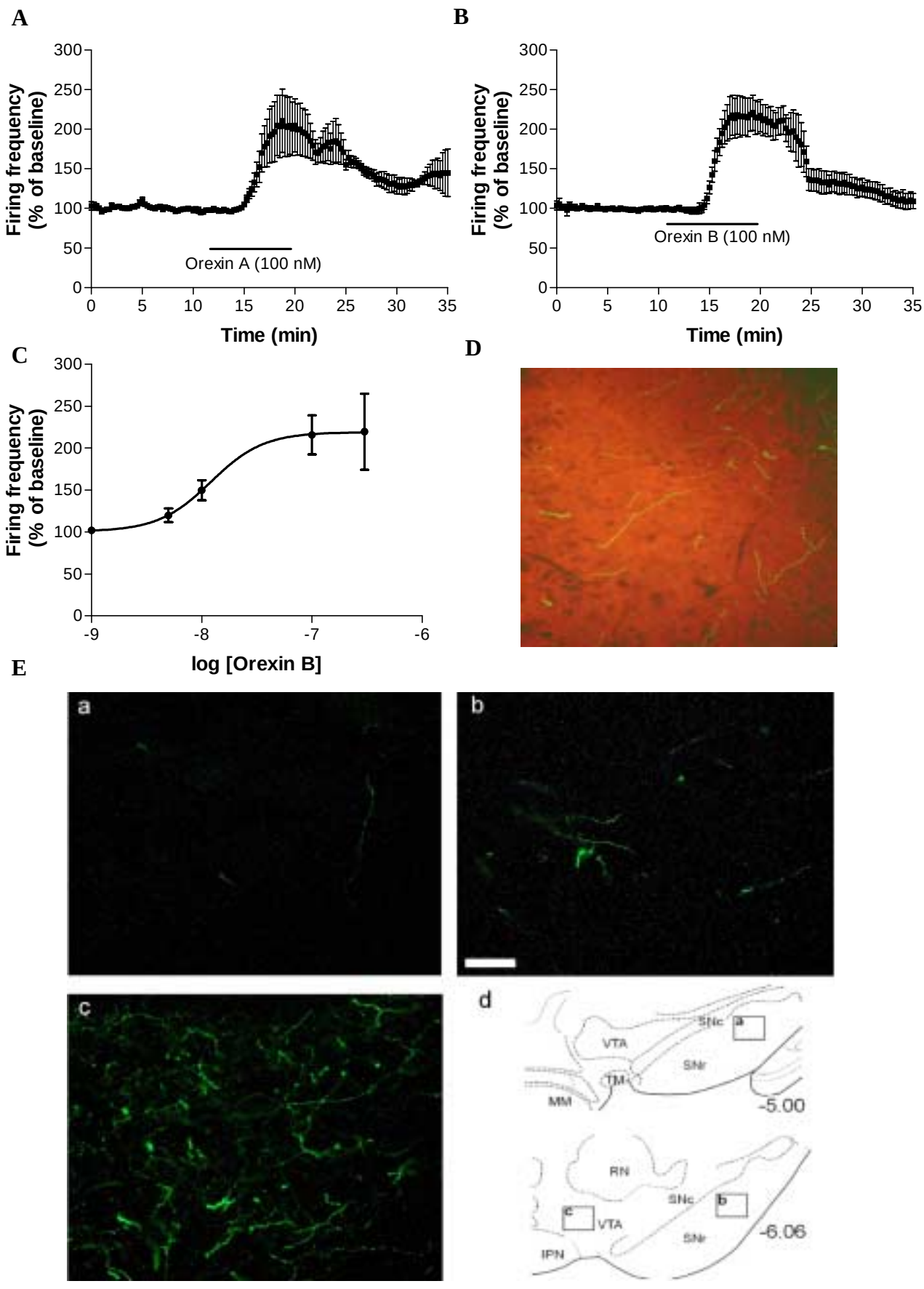

A
B
C
D
E
Firing frequency (% of baseline)
Time (min)
Orexin A (100 nM)
Orexin B (100 nM)
log [Orexin B]
a
b
c
d
SNc
VTA
SNr
TM
MM
-5.00
RN
IPN
-6.06

Figure 8. Orexins uniformly excite GABAergic cells in substantia nigra

A. Application of orexin A (100 nM) led to a large increase in the firing frequency of GABAergic neurons in SN pars reticulata to 204 ± 36 % of baseline. **B.** The application of orexin B also led to a large and dose-dependent increase in the firing frequency of GABAergic neurons in SN pars reticulata. **C.** Dose-response relationship. EC_{50} was 12 nM and Hill slope 1.8. At least 4 neurons were tested at 1, 5, 10, 100 and 300nM. **D.** Orexin immunolabeling showed the presence of orexin fibres (green) in substanta nigra pars reticulata (red).

E. Staining demonstrating the presence of orexin fibres in substantia nigra pars reticulata. The density of immunoreactive fibres is very low in the rostral substantia nigra (a) while the density is clearly higher in the caudal parts (b). The much higher density of orexin fibres in the ventral tegmental area is shown in (c). In (d) the locations of (a-c) are indicated. The rostrocaudal level is given as the distance from bregma. IPN, interpeduncular nucleus; MM, medial mammillary nucleus, RN, red nucleus, SNc/r, substantia nigra compacta/reticulata; TM, tuberomammillary nucleus; VTA, ventral tegmental area. Scale bar: 50 µM.

16.2.4. Effects of orexins on GABAergic neurons in VTA

In the VTA two groups of GABAergic cells were found (**Figs. 4D, 4F**). Both groups of presumed GABAergic cells were excited by orexins. In extracellular recordings, application of orexin A (100 nM) to the fast firing cells caused an increase in the firing frequency to 154 ± 21 % of baseline (n=10, $p<0.005$, **Fig. 9B**). Orexin B (100 nM) also increased the firing of these cells (n=5, $p<0.01$, 149 ± 29 %, not significantly different from orexin A). In intracellular recordings, the application of orexins caused a depolarization of 3.7 ± 0.3 mV (n=3, **Fig. 9A**). A similar amount of depolarization (3.9±0.4mV) was seen in the presence of TTX (n=3, **Fig. 9C**).

In extracellular recordings from the slow-firing GABAergic cells application of orexin A (100 nM) caused a very large increase in firing rate to 700 ± 171 % of baseline (n=6, $p<0.0005$, **Fig. 9E**). Orexin B increased the firing rate to 570 ± 113 % of baseline (**Fig. 9F**, n=3, not significantly different from orexin A). After washout of orexins and stabilization of the firing rate, the frequency of firing was still higher than before application of orexins, and did not return to the baseline level during 1 h of washout. In intracellular recordings orexin A (100 nM) caused a depolarization of 6 ± 2 mV (n=4, $p<0.05$). The depolarization was not blocked by tetrodotoxin (0.5 µM, n=2).

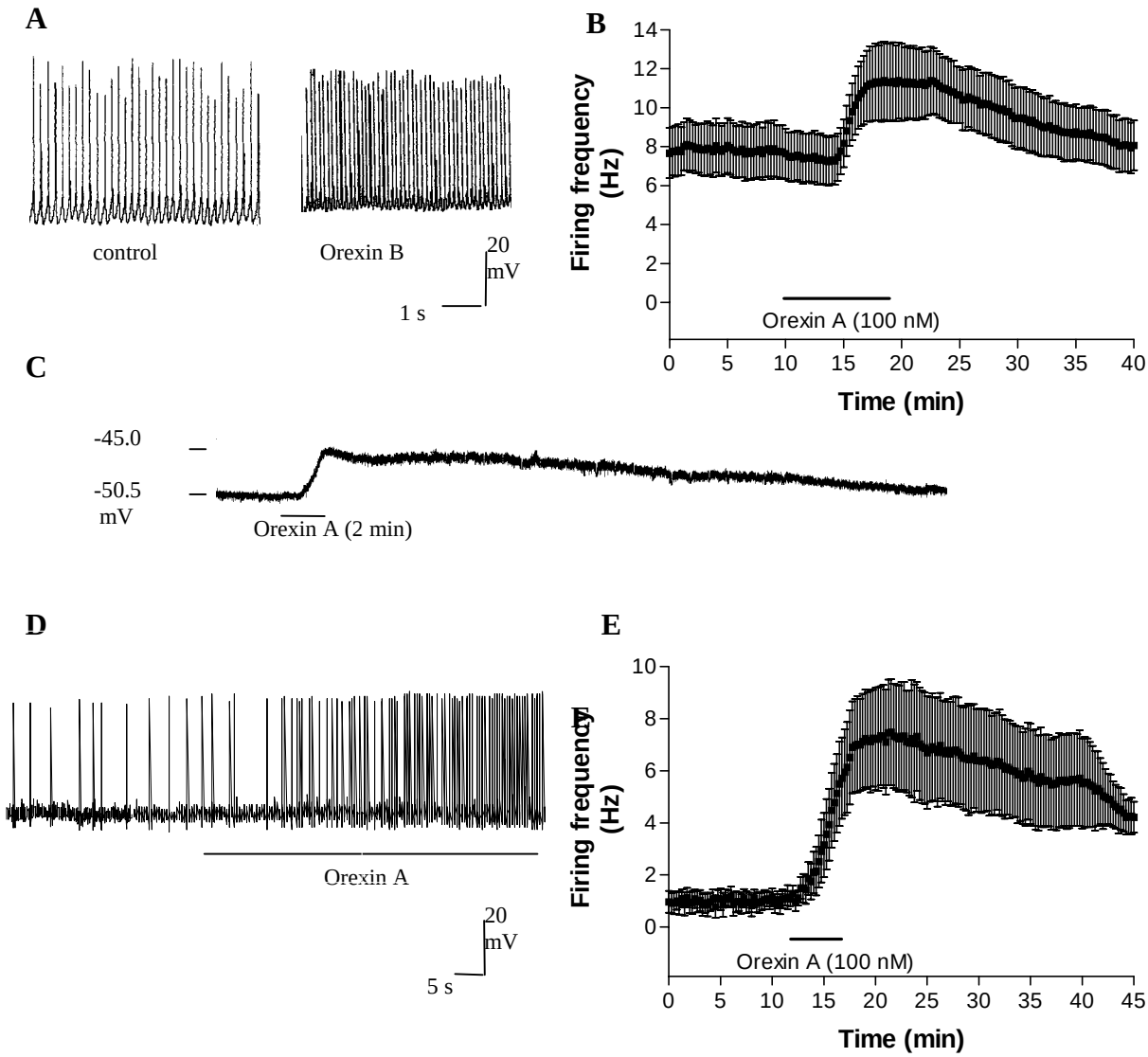


Figure 9. Effects of orexins on GABAergic neurons in VTA

A. Chart recording of membrane potential and spontaneous action potentials of the fast-firing GABAergic neuron before and after application of orexin B (100nM).

B. Application of orexin A (100 nM) to the fast firing cells caused an increase in the firing frequency to 154±21 % of baseline.

C. Chart recording of the membrane potential of the neuron after application of 0.5μM of TTX. Orexin A causes depolarization of the cell.

D. Chart recording of membrane potential and spontaneous action potentials before and after application of orexin A in slow-firing GABAergic neurons (100nM).

E. In the slow-firing GABAergic cells application of orexin A (100 nM) caused a very large increase in firing rate to 700±171 % of baseline.

16.2.5. Signal transduction mechanism of the orexin-induced excitation of GABAergic neurons in SN and VTA.

In further experiments we investigated the mechanism of the orexin-induced excitation of GABAergic SN neurons. This part of the study is summarized in **Table 3**. None of the inhibitors of signal transduction tested affected the firing rate of pars reticulata or ventral tegmental area cells on their own. The effect of orexin B (10 nM, **Fig. 10A**) was not affected by blocking synaptic transmission (using low (0.2 mM) Ca^{2+} / high (4 mM) Mg^{2+} solution), indicating a direct postsynaptic action on GABAergic cells (n = 4). Bath application of thapsigargin (1 µM) reduced significantly ($p < 0.01$) the effect of orexin B (10 nM) on GABAergic cells, indicating that calcium release from thapsigargin- sensitive intracellular calcium stores is an important component of the orexin - induced increase of firing rate (n = 6, **Fig. 10B**). In the presence of the broad-spectrum kinase inhibitor, H7 (100 µM), the effect of orexin B (10 nM) was significantly decreased (p< 0,005, n = 7, **Fig. 10C**). However, application of chelerythrine (10 µM), a specific inhibitor of protein kinase C, did not block the response to orexin B, suggesting that protein kinase C does not play a crucial role in the effect of orexin (n = 7, **Fig. 10D**). H7 is also an effective blocker of protein kinases A and G. Therefore, we tested the effect of H89, which at a concentration of 1 µM, shows selectivity for protein kinase A (IC_{50} 0.048 nM) and protein kinase G (IC_{50} 0.48 µM) over protein kinase C (IC_{50} 32 µM). At this concentration H89 blocked the effect of orexin B (10 nM, n = 3, p< 0.05). H89 was also effective at a ten-fold lower concentration (0.1 µM), suggesting a role for protein kinase A (n = 6, p< 0.05, **Fig. 10E**). To test this more directly we utilized the highly selective protein kinase A inhibitor KT5720. This drug, applied at a concentration of 1 µM almost completely blocked the action of 10 nM orexin B (n = 6, p < 0.005, **Fig. 10F**).

The effects of protein kinase inhibitors in VTA were also investigated using extracellular recordings. We found that the orexin A-mediated increase in firing rate of fast-firing GABAergic cells was not affected by the protein kinase C inhibitor, chelerythrine (n=4) but was significantly reduced by the selective protein kinase A inhibitor KT5720 (n=5, p<0.05). The orexin-mediated depolarization and increase in firing rate in DAergic cells were not significantly reduced by application of chelerythrine (10 µM, n=4) or KT 5720 (1µM, n=4).

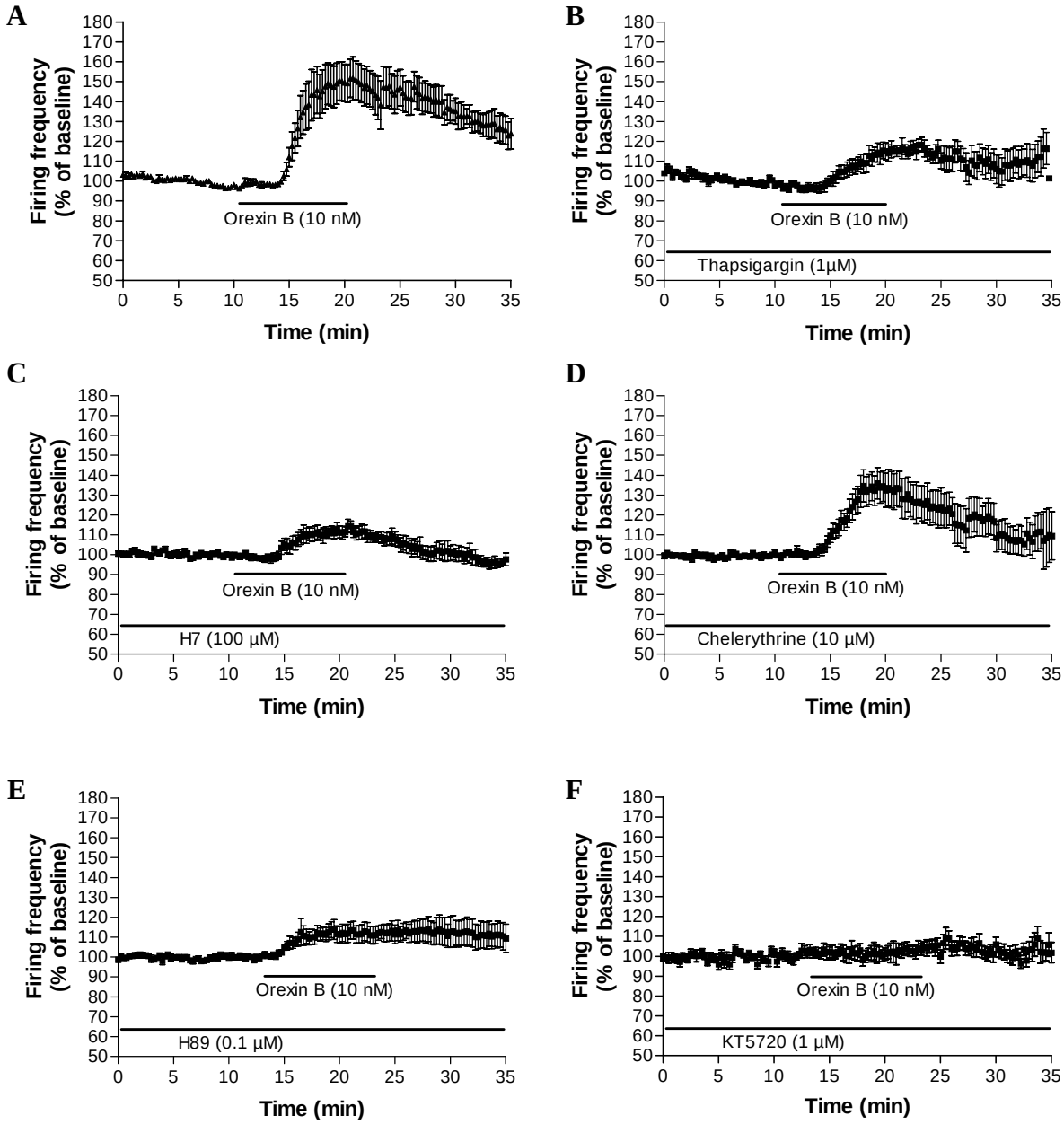


Figure 10. Signal transduction mechanism of the orexin B excitation of pars reticulata cells.

A. Effect of orexin B (10 nM) in control (n = 8).

B-F. Effect of orexin B in the presence of various inhibitors of signal transduction. The effect of orexin was reduced by the inhibitor of IP_3-sensitive stores, thapsigargin (**B**, n = 6), by the broad-spectrum kinase inhibitor H7 (**C**, n = 7) and by selective inhibitors of protein kinase A, H89 (**E**, n = 6) and KT5720 (**F**, n= 6) but not by the selective protein kinase C inhibitor, chelerythrine (**D**, n = 7).

Table 3. The inhibition of orexin-induced excitation.

Cell subtype	N of experim.	substance	concentration	Orexin(conc.)	Eff (P value)
GABA SN	4	low (0.2 mM) Ca^{2+} / high (4 mM) Mg^{2+} solution		B (10 nM)	No effect
GABA SN	6	thapsigargin	1µM	B (10 nM)	$p < 0.01$
GABA SN	7	H7	100µM	B (10 nM)	$p< 0.005$
GABA SN	7	chelerythrine	10µM	B (10 nM)	No effect
GABA SN	3	H89	1µM	B (10 nM)	$p< 0.05$
GABA SN	6	H89	0.1µM	B (10 nM)	$p< 0.05$
GABA SN	6	KT5720	1µM	B (10 nM)	$p < 0.005$
GABA VTA	5	KT5720	1µM	A (100 nM)	$p< 0.05$
GABA VTA	4	chelerythrine	10µM	A (100 nM)	No effect
DA VTA	4	thapsigargin	1µM	A (100 nM)	No effect
DA VTA	4	chelerythrine	10µM	A (100 nM)	No effect
DA VTA	4	KT5720	1µM	A (100 nM)	No effect

16.3. Electrophysiological differences between DAergic cells with different response to orexins

While the effect of orexins on GABAergic neurons and signal transduction mechanism appeared to be straight and clear, our findings concerning orexins action on DAergic neurons in VTA raised many questions. First, in contrast to GABAergic neurons, protein kinase A inhibitor KT5720 (1µM) did not block orexin-induced excitation in DAergic cells (n=4). Application of chelerythrine (10 µM), a specific inhibitor of protein kinase C, also did not block the response to orexins (n=4). Second, the effect of orexins was not uniform in the DAergic cells. No correlation between the position of the neuron in the VTA and its response to orexin was found. Third, the usual point of view on interaction between DAergic and GABAergic neurons in VTA suggests the counter-regulation between these neurons, excitation of GABAergic VTA system should lead to inhibition of the DAergic system, so substances were supposed to act the opposite, not the same way on these systems.

To refer to the first question, we investigated the possibility that the orexin effects are mediated via a recently identified family of nonselective cation channels, the transient receptor potential channels (TRPC). They are suggested to be coupled to phospholipase C and generate “noisy” currents (Brown et al., 2002). As far as TRPC receptor antagonists are not yet available, we could only study the expression of these channels in VTA by single-cell RT-PCR. These results are described below together with other RT-PCR data.

To address the question what other possible differences could exist between DAergic neurons which have different responses to orexins, we analysed the electrophysiological characteristics of these cells (**Table 4**).

Table 4. The electrophysiological characteristics of neurons with different responses to orexins.

	no effect of orexins (n=4)	depolarization and incr.in fir. rate (n=5)	oscillations / burst firing (n=5)
spike threshold	47 ±3 mV	43.4 ±1.7 mV	45.8 ±2.4 mV
firing frequency	4 ±0.5 Hz	3.4 ±0.2 Hz	3.8 ±0.5 Hz
I_{AHP}	17.3 ±0.8 mV	17.8 ±1.3 mV	12.8 ±1.8 mV

As we can see from these data, the only significant difference between these groups is the AHP amplitude in neurons that show oscillatory firing in response to orexin. In comparison with two other groups it had a smaller I_{AHP} (AHP amplitude was 12.8 ± 1.9 vs 17.6 ± 0.8 mV, $p<0.005$). Recently, it has been demonstrated in mice that different functional groups of TH-positive neurons can be discriminated in the VTA according to the expression of calbindin; interestingly, calbindin-positive DAergic cells had smaller afterhyperpolarizations than calbindin-negative neurons (Neuhoff et al., 2002).

16.4. Single-cell RT-PCR from acutely isolated VTA cells

The cytoplasm was extracted from 39 acutely isolated VTA neurons (from 10 rats, Wistar, 3-4 weeks old) and single-cell PCR performed for TH, glutamic acid decarboxylase (GAD), orexin receptors (OX_1 and OX_2), calbindin (CB) and neuropeptide Y (NPY) mRNA (**Fig. 11B**). Total mRNAs isolated from ventral tegmental area (VTA) was used as positive control. Isolated neurons could be assigned to three groups based on their expression of tyrosine hydroxylase (TH) and or glutamic acid decarboxylase (GAD). Results of RT-PCR study are summarized in **Table 5**.

Table 5. Distribution of mRNA for tyrosine hydroxylase (TH), glutamatic acid decarboxylase (GAD), orexin receptors (OX_1 and OX_2), calbindin (CB) and neuropeptide Y (NPY) in VTA neurons.

		$OX_1^+OX_2^+$			$OX_1^+OX_2^-$			$OX_1^-OX_2^+$			$OX_1^-OX_2^-$		
	Soma Size(μm)	N	CB^+	NPY^+	N	CB^+	NPY^+	N	CB^+	NPY^+	N	CB^+	NPY^+
TH^+GAD^- (n=21)	22.5±0.8	9	6	4	7	5	4	0	0	0	5	0	1
TH^+GAD^+ (n=5)	21.2±1.0	1	0	1	3	0	1	1	0	1	0	0	0
TH^- (n=13)	16.3±1.0	4	1	0	5	3	0	1	1	1	4	0	1

The first group of neurons (n = 21) was TH-positive and GAD-negative. These cells were 22.47 ± 0.84μM in diameter and had polygonal or round shapes (**Fig. 11A**). Nine of these 21 neurons expressed both types of orexin receptors (**Fig. 11B**); four of them (44.4%) expressed NPY, 6 cells (66.7%) expressed CB (3 of them colocalized CB and NPY). Seven neurons expressed OX1 alone and none expressed OX2 alone. Five of them (71.4%) expressed CB; four expressed NPY. Two neurons coexpressed CB and NPY. Five TH positive, GAD-negative neurons did not express either orexin receptor. These cells also lacked calbindin. One cell expressed NPY.

Thus, CB was expressed only in cells, which express at least one orexin receptor. CB^+ /TH^+ positive cells had a soma size of 20.3 ± 0.3 μm (n=11) which was significantly smaller than for CB^-/TH^+ negative cells (23.9 ± 1.2 μm, n=15, $p<0.05$).

The second group of cells was positive both for TH and GAD (n = 5, **Fig.11A**). Their average soma size was 21.2 ± 1.02μm. All of them expressed at least 1 type of orexin receptor (1 both, 2 orexin type 1,1 only orexin 2). One neuron from each group (n=3) coexpressed NPY; none coexpressed calbindin.

The third group consisted of TH-negative, GAD positive cells (n =2) or those with similar properties, in terms of size and spike width (n = 11, spike widths 1.3 ± 0.1 ms). Their soma size was 16.3 ± 1.03μm (significantly smaller than in TH^+ cells; p=0.0001).

Four cells had both receptors (1 expressed CB as well, none NPY), four had only type 1 (3 coexpressed CB, 1 only type 2, it expressed CB and NPY as well). Four cells lacked orexin receptors and CB, 1 of them expressed NPY. TH-negative neurons, which expressed at least 1 type of orexin receptor were smaller (14.8 ± 1.1 μm) than TH-negative cells that did not express orexin receptors (19.5 ± 1.0 μm, $p<0.02$).

Hence, we found that calbindin (CB) was only expressed in cells, which express at least one subtype of orexin receptor and was never expressed in TH-positive cells, which were also positive for GAD. CB was expressed in 52.4 % (11/21) of TH-positive cells and 55.6 % (5/9) of TH-negative cells that expressed orexin receptors and was never detected in cells that lacked orexin receptors. In contrast, there was no relationship between the expression of NPY and orexin receptors or NPY and calbindin.

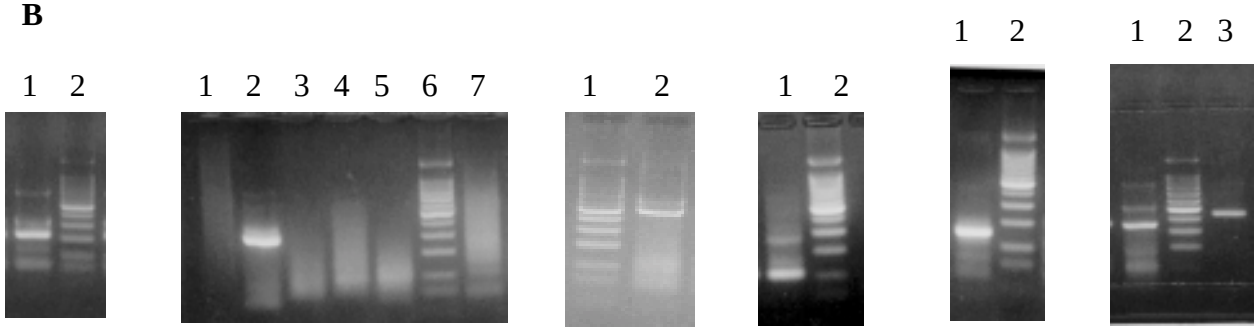
The expression of TRPC channels was studied on isolated neurons from 12 rats (Wistar, 3-4 weeks old). The majority of TH-positive cells (73%, 8/11) in VTA expressed TRPC channels (**Fig.11**). TRPC1, TRPC2, TRPC4 and TRPC6 isoforms were coexpressed in 3 dopaminergic neurons, the rest of the cells lacked one or more of them. TRPC3, TRP5 and TRPC7 subunits were never detected in TH-positive neurons. TH-negative, GAD65/67-positive VTA neurons, expressed TRPC channel in two of five neurons. The pattern of expression differed from that seen in dopaminergic neurons: one neuron expressed only the TRPC3 subunit, while the other possessed TRPC1, TRPC5 and TRPC6 mRNAs. In VTA neurons the expression of the two orexin receptors did not correlate with the presence or absence of TRPC subunits. Two of the TRPC-positive neurons in VTA did not express orexin receptors; three OX1-positive cells in VTA, of which two possessed the OX2 receptor as well, expressed none of the TRPC subunits. In contrast, in dorsal raphe, which is also activated by orexins (Brown et al., 2001a, Brown et al., 2002) a coordinated expression of orexin receptors and TRPC subunits was observed: all studied neurons (n=8) expressed the orexin receptor 1 (OX1) and the TRPC6-channel subunit, 75% of them expressed OX2 and at the same time the TRPC5 subunit (two cells lacked expression of OX2 and TRPC5). The study of two other aminergic nuclei which are excited by orexins – tuberomamillary nucleus (TM) and locus coeruleus (LC) revealed that in TM there was no correlation between expression of orexin receptors and TRPC subunits (n=15) and in LC no TRPC channels were detected but all studied neurons expressed OX1 (n=12), one neuron expressed OX2 as well (Sergeeva, Korotkova et al., 2003).

A

TH+ GAD- (n=21) | TH+ GAD+ (n=5) | TH-GAD+ (n=13)

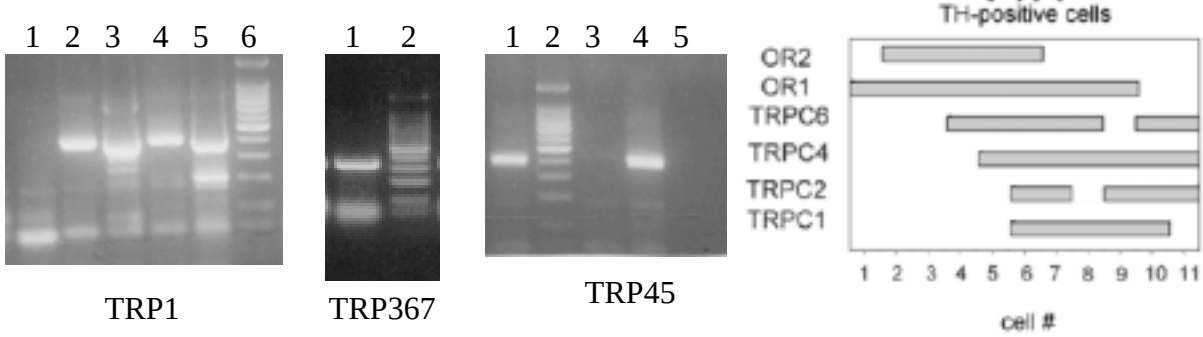
22.5±0.8 µm | 21.2±1.0 µm | 16.3±1.0 µm

B



TH | GAD65 | GAD67 | OX1 | OX2 | NPY, CB

C



TRP1 | TRP367 | TRP45

D

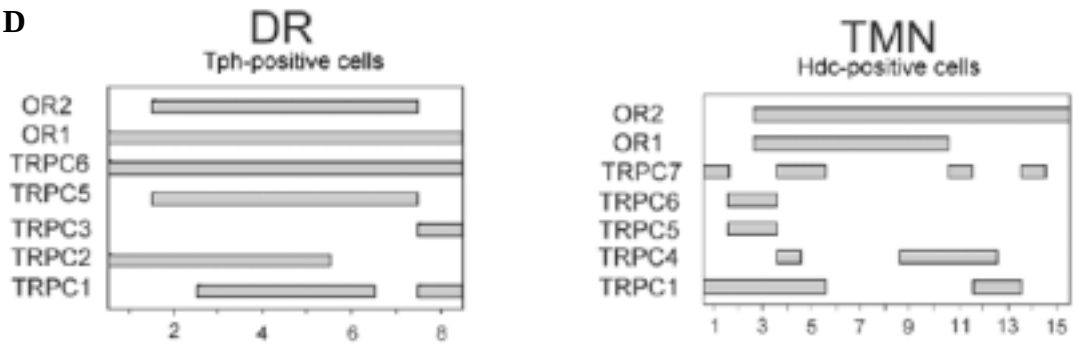


Figure 11. Representative results from single-cell RT-PCR study.

A. Video images of typical dissociated neurons from each subgroup.

B,C. The results of amplification of mRNA from several single cells. Single neurons were tested for the expression of tyrosine hydroxilase (TH), glutamic acid decarboxylase 65 (GAD65) and 67 (GAD67), orexin 1 (Or1), orexin 2(Or2), neuropeptide Y (NPY), calbindin (CB) and subunits of transient receptor potential canonic (TRPC) channels. TH: 1-TH-positive cell, 2-weight marker (WM, 100-basepairs-step DNA-bladder (Promega) with the 500 b.p. band present at triple intensity); GAD65 – only cell 2 is positive, 6-WM; GAD67 – 1-WM,2-positive; Or-1- 1-WM,2-positive; NPY,CB- 1-NPY-positive, 2-WM, 3-CB-positive; TRP1- 2-5-positive,6-WM; TRP367-1-positive, 2-WM; TRP45-1,4-positive,2-WM.

C,D. Expression patterns of the two orexin receptors (OR1 and OR2) and TRPC channels in individual neurons from three different structures: VTA, dorsal raphe (DR) and tuberomamillary nucleus (TMN). Bar plots illustrate overlapping expression of TRPC subunits and orexin receptors in 8 DR, 15 TM and 11 VTA neurons.

16.5. Application of orexins did not affect field potentials in ventral and dorsal striatum

Neither orexin A nor orexin B (500nM) changed negative-going components of field potentials in the core accumbens (n=5), shell accumbens (n=5) or dorsal striatum (n=6), referred to as N1 and N2 (**Fig.12**). Orexin B was appled only in two recordings (from shell accumbens and dorsal striatum), afterwards only orexin A was applied, because it is an agonist of both orexin receptors. The N1 component represents direct activation of medium spiny neurons, whereas the N2 component is synaptically mediated. This finding suggests that orexins do not affect striatal transmission or excitability directly but could modulate the activity in ventral striatum via VTA neurons.

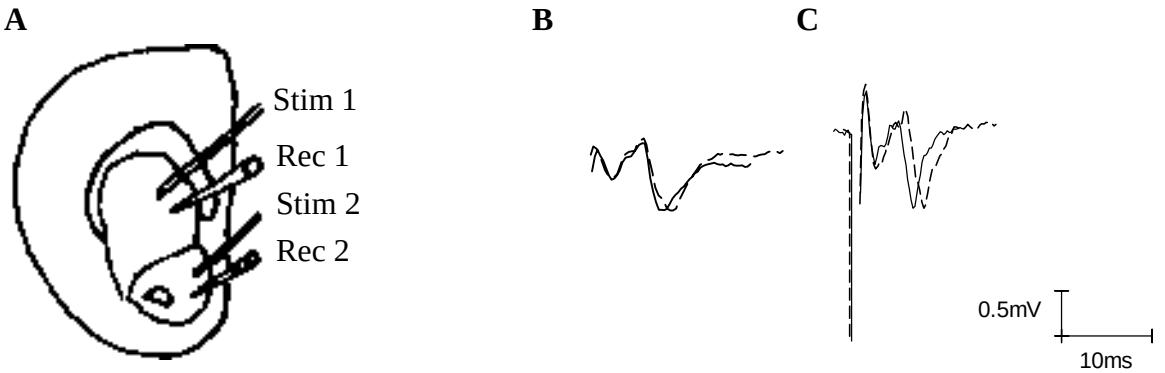


Figure 12. Orexins did not affect field potentials in ventral and dorsal striatum.

A. Diagram showing the position of stimulating and recording electrode. Stimulating and recording electrodes were placed either in dorsal or in ventral striatum. Stimulation within the dorsal striatum (Stim 1) elicited a field potential detected with a recording electrode in dorsal striatum (Rec 1) and likewise for the nucleus accumbens (ventral striatum) (Stim2, Rec2).**B, C.** Bath application of orexins did not affect significantly the amplitude on N1 and N2 components in dorsal (B) and ventral (C) striatum.

16.6. Effects of hypothalamic feeding- and arousal-related peptides on VTA neurons

Could other feeding- or arousal-related peptides also affect the DAergic and GABAergic midbrain systems? The following neuropeptides were studied: melanin-concentrating hormone (MCH), which is also expressed exclusively in the perifornical hypothalamic area, and has a similar action on feeding; neuropeptide Y (NPY), a very potent stimulator of food intake; the orexigenic neuropeptides ghrelin and agouti-related protein (AGRP), α-melanocyte stimulating hormone (α-MSH), hypothalamic anorectic neuropeptide; corticotropin-releasing factor (CRF), another anorectic peptide which is also known to promote arousal; anorectic cocaine and amphetamine-related transcript (CART) and substance P (SP) that has little effect on food intake but promotes arousal. Effects of these peptides are summarized in **Table 6**.

16.6.1. Effects of orexigenic neuropeptides on VTA neurons

16.6.1.1. Responses to melanin concentrating hormone

Effects of melanin-concentrating hormone (MCH) were studied only in drug-naive slices. MCH (100 nM) did not affect the firing frequency of DAergic (n = 4) or GABAergic (n = 4) neurons in the SN. In VTA MCH (100 nM) failed to affect the firing of dopaminergic (n=4) or fast-firing GABAergic cells (n=3). The application of 1μM of MCH (n=7 for DAergic and n=6 for GABAergic cells) also did not affect membrane potential and firing rate of the cells.

16.6.1.2. Responses to neuropeptide Y.

In contrast to the previous sections of study where actions of only one neuropeptide on one neuron were studied, in the following sections (16.6.1.2.- 16.6.3) effects of several neuropeptides on the same cell were tested by extracellular single-unit recordings: after recording of the baseline for 15 min the first peptide was applied, after the end of the effect (return of the baseline to the initial value) and a stable baseline for the next 15 minutes the second drug was applied and so on. The order of drug application was changed each time to

be sure that the previous application did not affect the next one, the action of each neuropeptide in drug-naïve cells (in whole-cell intracellular recordings) was also tested. Extracellular recordings of 30 DAergic neurons, 16 fast-firing GABAergic neurons and 6 slow-firing GABAergic neurons were performed (as in previous parts of the study, only one cell per slice was recorded, in all but four cases one slice from one rat was cut, one DAergic and one GABAergic cell from different slices of these four rats were recorded). An overview of the order of drugs application is presented in **Tables 7**, **8** and **9**. Repetitive applications of the same drug (higher doses in unresponsive cells) and application of dopamine or DAMGO that, as always, took place at the end of each experiment, are not shown in these tables.

NPY (300nM, in unresponsive cells higher doses (1μM) were also tested (n=4)) inhibited 40% of DAergic cells, recorded extracellularly (7 out of 19, **Fig. 13A**), two of them – completely, others – up to 68,1 ± 15,6 % of control rate ($p < 0.01$). Two NPY-inhibited neurons and four cells that were not affected by NPY were drug-naive. Intracellular recordings in drug-naive rats revealed that reduction in firing frequency was accompanied by a moderate hyperpolarization (2,5mV; 3 mV, n=2, **Fig. 13C**). In six NPY-inhibited cells, which were tested for response to orexins as well, all neurons were excited by orexins. However, other five neurons, which were also tested for both neuropeptides and were not affected by NPY, were excited by orexins.

In fast firing GABAergic cells, NPY inhibited 6 out of 11 neurons by up to 55,6 ± 13,8% ($p < 0.005$, **Fig. 13B**), the firing rate of 5 neurons was not changed. Two affected and one – unaffected neurons were drug-naive. Both slow-firing GABAergic cells tested were inhibited by NPY (one drug-naive).

Additional recordings of slow-firing GABAergic neurons should be performed to study NPY action on this subgroup. Additional whole-cell recordings after the application of tetrodotoxin should be performed in DAergic and GABAergic neurons to study if the effect of NPY on both groups is direct.

16.6.1.3. Responses to ghrelin

Ghrelin (500 nM) did not affect firing rate and membrane potential of DAergic (n=8), fast (n=4) and slow (n=2) GABAergic VTA neurons. Two DAergic and two GABAergic neurons were drug-naive before the application of ghrelin.

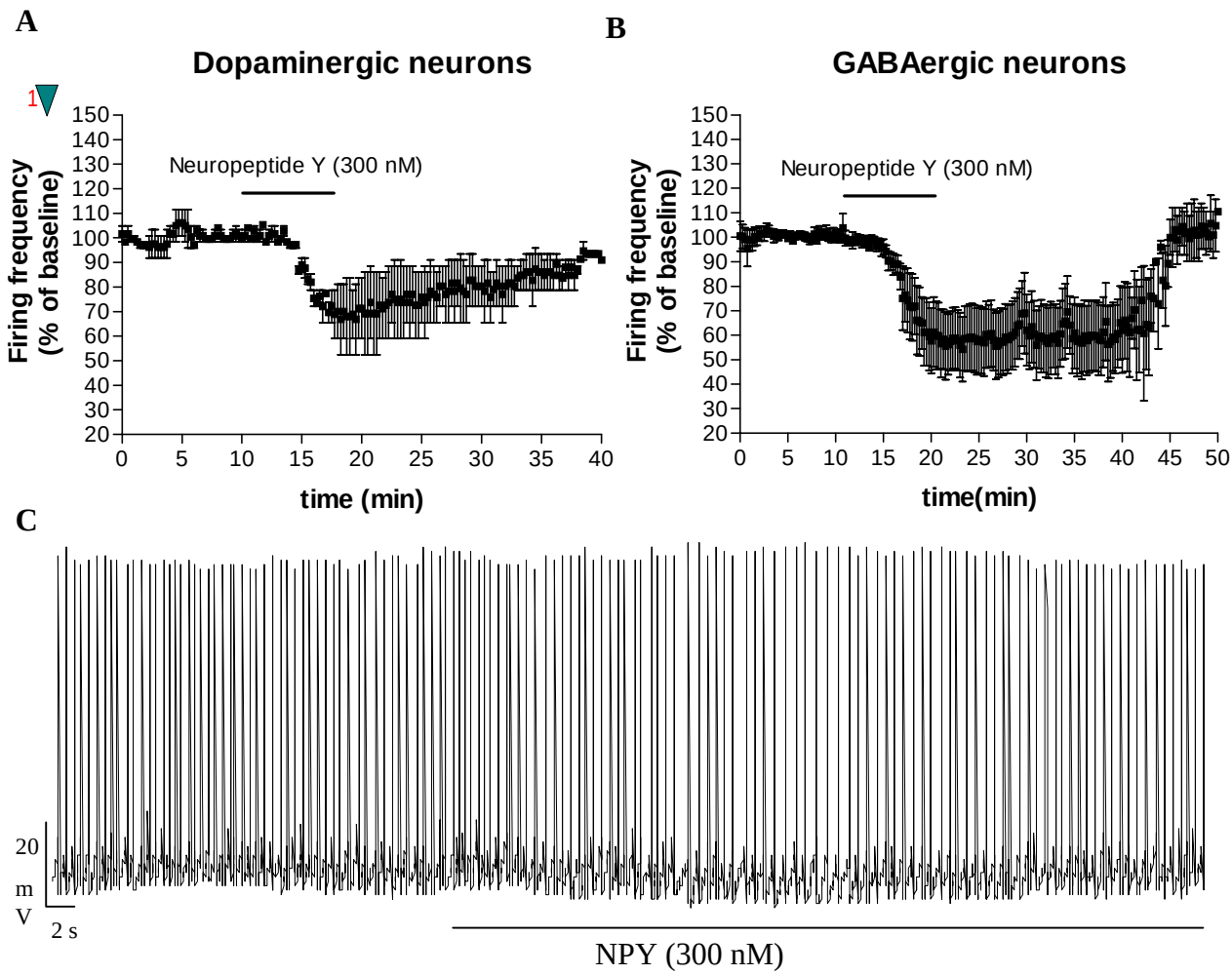


Figure 13. Neuropeptide Y (NPY) inhibited a subgroup of DAergic and GABAergic neurons.

A. NPY inhibited 40 % of DAergic cells in VTA.

B. In fast-firing GABAergic cells, NPY reduced the firing rate of 54.6% of neurons.

C. Whole-cell patch-clamp recording of membrane potential and spontaneous action potentials of the DAergic VTA neuron before and after application of NPY (300nM).

16.6.1.4. Responses to agouti-related protein (AGRP)

AGRP (1μM) did not affect DAergic (n=4) and GABAergic (n=5) VTA neurons. Two DAergic and two GABAergic neurons were drug-naive before the application of AGRP.

16.6.2. Effects of anorectic neuropeptides on VTA neurons.

16.6.2.1. Responses to α-melanocyte stimulating hormone.

α-MSH had no effect on firing rate or membrane potential of DAergic cells (500nM, n=6, 1μM, n=6), three of these cells were drug-naive, but excited 3 out of 11 GABAergic cells (500nM) and reduced the firing rate of 1 GABAergic neuron. Two α-MSH-excited, 1 α-MSH-inhibited and 3 GABAergic neurons which were not affected by this neuropeptide, were drug-naïve. Two GABAergic neurons, excited by α-MSH, which were tested for NPY (300 nM), were inhibited by it. Additional experiments in drug-naive GABAergic neurons, especially whole-cell recordings in the presence of tetrodotoxin, will clarify the action of α-MSH on GABAergic VTA neurons.

16.6.2.2. Responses to corticotropin-releasing factor (CRF).

Bath application of CRF (200nM) excited 63.6% (7 out of 11) of DAergic neurons up to 161.6 ± 13.4% ($p<0.001$, **Fig.14A**); two CRF-excited cells and one neuron that was not affected by CRF, were drug-naive; additional whole-cell recordings in other drug-naïve DAergic neurons revealed that the increase in firing rate was accompanied by a depolarization (4,5 mV, 4mV, n=2, **Fig.14C**); it increased the firing rate in all tested fast-firing GABAergic neurons (n=6, 132.1±7.1%, $p<0.005$, **Fig.14B,** one cell was drug-naive) and slow-firing GABAergic neurons (n=3, 370±152%, one neuron was drug-naive) as well. As in previous sections, intracellular recordings in the presence of tetrodotoxin should be performed to study whether the CRF-induced excitation is mediated by direct postsynaptic effect of this neuropeptide. The number of experiments in drug-naive cells should also be increased.

16.6.2.3. Responses to cocaine and amphetamine-related transcript

CART (500 nM) did not affect firing rate and membrane potential of DAergic (n=6, two drug-naïve) or GABAergic (n=7, four drug-naive) VTA neurons.

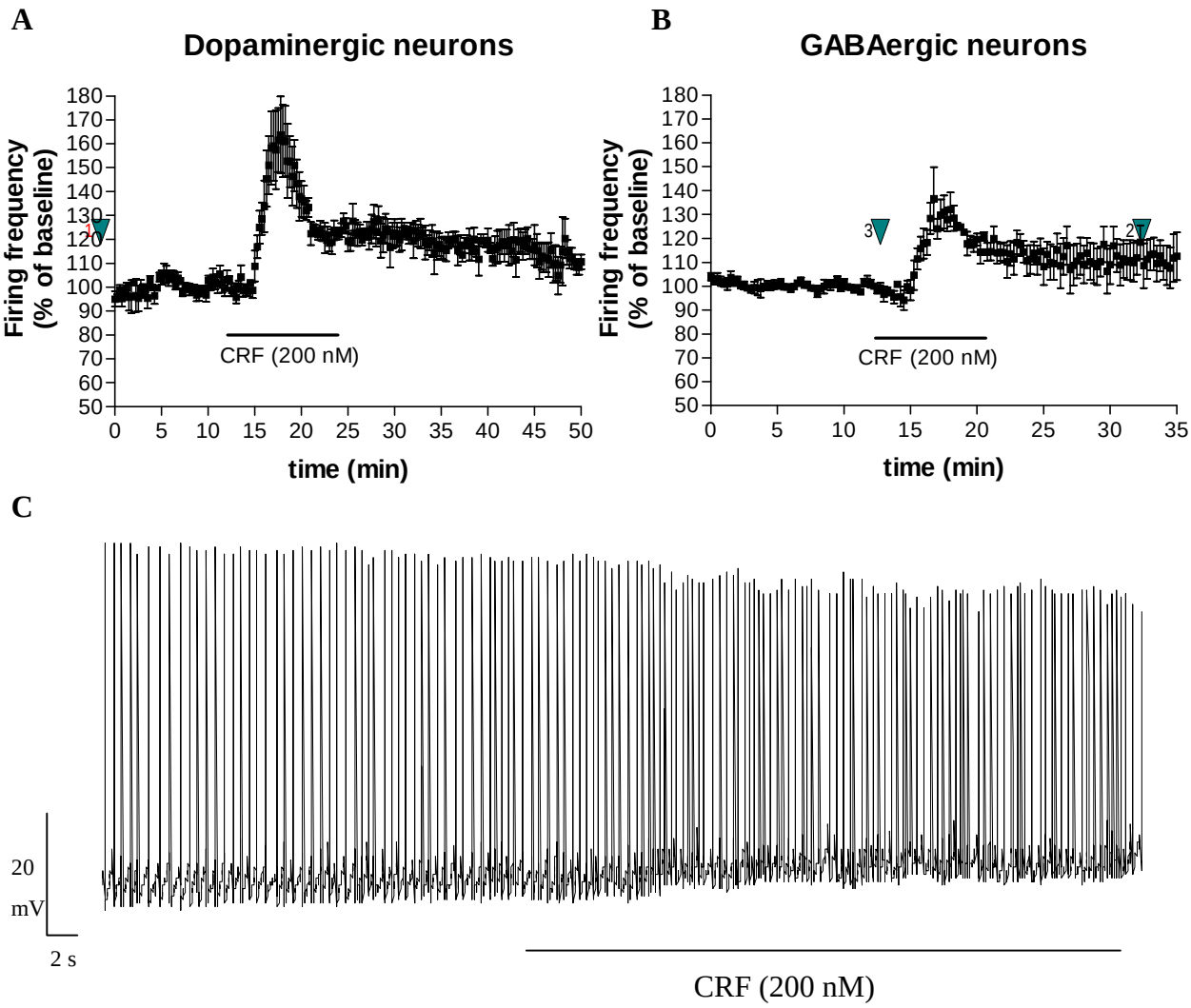


Figure 14. Corticotropin-releasing factor (CRF) excited DAergic neurons in VTA.
A. Bath application of CRF (200nM) leads to increase in firing rate of DAergic neurons.
B. CRF increases the firing rate of GABAergic VTA neruons as well.
C. CRF-induced increase in firing rate is accompanied by the depolarizatoin.

16.6.2.3. Responses to leptin

Leptin (500 nM, in 5 neurons the repetitive application of 1μM was made) had no effect on firing rate and membrane potential of DAergic (n=7, two drug-naive) or GABAergic (n=5) VTA neurons.

16.6.3. Responses to substance P

Bath application of SP (300 nM) increased the firing rate of the majority of VTA DAergic neurons up to 145.3±18.5% (**Fig. 15A**, 300nM, 9 out of 11 were excited, $p<0.0005$, two SP-excited cells and one cell that was not affected by SP, were drug-naive) and depolarized intracellularly recorded neuron (3 mV, n=1, **Fig. 15C**). Six neurons excited by SP were excited by orexin as well, no relationship with the effect of NPY on the same cells was found. All tested fast-firing GABAergic cells (n=5, two drug-naïve) were excited by SP up to 139.0±14.8% ($p<0.001$).

Both tested slow-firing GABAergic cells (one was drug-naive) were excited by SP (432%; 396%) as well.

Table 6. Effects of hypothalamic neuropeptides on VTA neurons.

Cell subtype	Neuropeptide	Concentration	Neurons affected/ studied	Effects on the firing rate, %
DA SN	MCH	100 nM	0/4	No effect
GABA SN	MCH	100 nM	0/4	No effect
DA VTA	MCH	100 nM	0/4	No effect
DA VTA	MCH	1μM	0/7	No effect
GABA f.f. VTA	MCH	100 nM	0/3	No effect
GABA f.f. VTA	MCH	1μM	0/6	No effect
DA VTA	NPY	300 nM	7/19	68,1 ± 15,6 %
DA VTA (unresp.for lower dose)	NPY	1μM	0/4	No effect
GABA f.f. VTA	NPY	300 nM	6/11	55,6 ± 13,8%
GABA s.f. VTA	NPY	300 nM	2/2	Comlete inhib.
DA VTA	Ghrelin	500 nM	0/8	No effect
GABA f.f. VTA	Ghrelin	500 nM	0/4	No effect
GABA s.f. VTA	Ghrelin	500 nM	0/2	No effect
DA VTA	AGRP	1μM	0/4	No effect
GABA VTA	AGRP	1μM	0/5	No effect
DA VTA	α-MSH	500 nM	0/6	No effect
DA VTA	α-MSH	1μM	0/6	No effect
GABA VTA	α-MSH	500 nM	4/11	Contr.eff.(see text)

DA VTA	CRF	200 nM	7/11	161.6 ± 13.4%
GABA f.f. VTA	CRF	200 nM	6/6	132.1±7.1%
GABA s.f. VTA	CRF	200 nM	3/3	370±152%
DA VTA	CART	500 nM	0/6	No effect
GABA VTA	CART	500 nM	0/7	No effect
DA VTA	Leptin	500 nM	0/7	No effect
GABA VTA	Leptin	500 nM	0/5	No effect
DA VTA	Substance P	300 nM	9/11	145.3±18.5%
GABA f.f. VTA	Substance P	300 nM	5/5	139.0 ±14.8%
GABA s.f. VTA	Substance P	300 nM	2/2	432%; 396%

f.f. – fast-firing,
s.f. – slow-firing.

Table 7. The order of application and effects of orexigenic and anorectic neuropeptides on DAergic VTA neurons.

N	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.
1	NPY	**no**														
2	ghr	**no**	OrB	**↑osc**												
3	αMSH	**no**	ghr	**no**	OrB	**no**										
4	αMSH	**no**	CART	**no**	OrA	**↑**	NPY	**↓**	OrB	**↑**						
5	OrA	**↑**	NPY	**no**												
6	NPY	**no**	αMSH	**no**	OrB	**no**										
7	OrA	**no**	NPY	**no**												
8	NPY	**no**	OrA	**↑**	AGRP	**no**										
9	αMSH	**no**	NPY	**no**	OrB	**↑osc**										
10	NPY	**no**	OrB	**no**												
11	CART	**no**	ghr	**no**												
12	OrB	**↑**	αMSH	**no**	AGRP	**no**										
13	OrA	**no**	NPY	**no**												
14	OrA	**↑**	αMSH	**no**	NPY	**no**	CART	**no**								
15	OrA	**↑**	NPY	**no**	SP	**↑**	CART	**no**	CRF	**no**	αMSH	**no**				
16	SP	**no**	NPY	**↓**	CRF	**↑**	αMSH	**no**	CART	**no**	OrA	**↑**				
17	SP	**↑**	CRF	**↑**												
18	OrA	**no**	SP	**↑**	αMSH	**no**	CRF	**↑**								
19	NPY	**↓**	CRF	**no**	SP	**↑**	αMSH	**no**	CART	**no**	OrA	**↑**				
20	CRF	**no**	SP	**↑**	OrA	**↑**	NPY	**↓**								
21	NPY	**↓**	αMSH	**no**	OrA	**↑**	lep	**no**	CRF	**no**	OrB	**↑**	SP	**↑**		
22	lep	**no**	CRF	**↑**	NPY	**no**	SP	**↑**	ghr	**no**	OrA	**↑**				
23	CART	**no**	ghr	**no**	CRF	**↑**	NPY	**↓**	OrA	**↑**						
24	CRF	**↑**	ghr	**no**	αMSH	**no**	SP	**↑**	NPY	**↓**						
25	CRF	**↑**	NPY	**no**	SP	**↑**	OrA	**↑**								
26	ghr	**no**	AGRP	**no**	lep	**no**										
27	AGRP	**no**	lep	**no**												
28	AGRP	**no**	ghr	**no**	lep	**no**										
29	lep	**no**														
30	SP	**no**	OrA	**no**	lep	**no**										

↑ - increase of the firing rate; ↓ - decrease of the firing rate; **no** – no effect.

Table 8. The order of application and effects of neuropeptides on fast-firing GABAergic VTA neurons.

N	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.
1	NPY	↓												
2	αMSH	**no**	NPY	**no**	ghr	**no**	OrB	↑						
3	NPY	↓	αMSH	↑	OrA	↑								
4	NPY	**no**	OrA	↑										
5	αMSH	**no**	NPY	**no**	OrB	↑								
6	ghr	**no**	αMSH	**no**	CRF	↑								
7	αMSH	↑	NPY	↓										
8	αMSH	↑												
9	αMSH	↓	CRF	↑	SP	↑	CART	**no**	lep	**no**	AGRP	**no**		
10	αMSH	**no**	CART	**no**	NPY	↓								
11	CRF	↑	αMSH	**no**	AGRP	**no**	CART	**no**	SP	↑	lep	**no**	NPY	**no**
12	CART	**no**	ghr	**no**	αMSH	**no**	SP	↑	CRF	↑	NPY	**no**	lep	**no**
13	CART	**no**	αMSH	**no**	NPY	↓								
14	SP	↑	CRF	↑	NPY	↓								
15	AGRP	**no**	SP	↑	CRF	↑								
16	SP	↑	αMSH	**no**										

↑ - increase of the firing rate;
↓ - decrease of the firing rate;
no – no effect.

Table 9. The order of application and effects of neuropeptides on slow-firing GABAergic VTA neurons.

N	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.	drug	eff.
1	NPY	↓	OrA	↑										
2	SP	↑	CART	**no**	ghr	**no**	OrA	↑	CRF	↑				
3	CART	**no**	CRF	↑	NPY	↓								
4	AGRP	**no**												
5	CRF	↑	SP	↑										
6	ghr	**no**	AGRP	**no**										

↑ - increase of the firing rate;
↓ - decrease of the firing rate;
no – no effect.

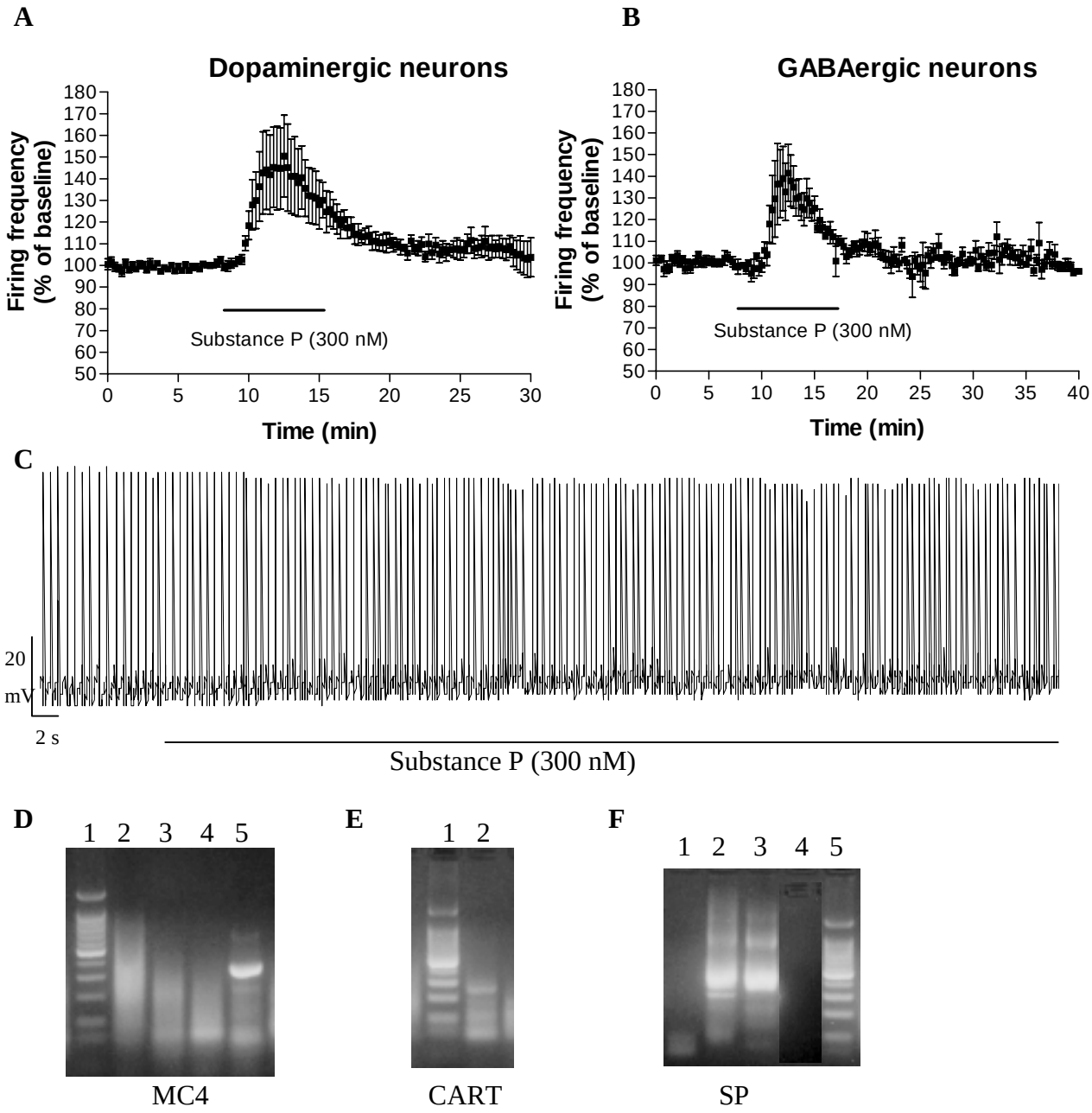


Figure 15. Substance P (SP) excited DAergic and GABAergic neurons in VTA. **A,B.** Bath application of SP (300nM) increased the firing rate of DAergic and GABAergic cells. **C**.An intracellular recording of DAergic neuron which was depolarized by the application of SP.
D, E,F – products of single-cell PCR amplification. **F** – cells 2,3 are positive for SP mRNA, **5-** weight marker; although no effect on membrane potential and firing rate was detected, mRNA for MC4 (**D**, cell 5) and CART (**E**, cell 2) were found in single neurons.

16.7. The expression of hypothalamic peptides and their receptors in isolated VTA neurons.

The RT-PCR experiments demonstrated the presence of mRNA for the described peptides or their receptors in the VTA (**Figs. 15 D,E,F**); The detailed analysis of expression of these proteins in single cells will allow us to find whether there is a relationship of their expression in a given neuron. In preliminary, pilot study it was not yet found, suggesting the mediation of effects through different neurons rather than a counter-regulation of these peptides via reciprocal effects on the same cells. However, additional expreriments should be performed to address this question.

Taken together, the effect on VTA cells could be linked to the influence of the given neuropeptide on arousal, rather than on food intake: NPY is the only substance among the tested neuropeptides that possesses anxiolytic properties and decreases wakefulness, and it's the only one that inhibits DAergic neurons. On the other side, SP that promotes activation but not food intake, excites the majority of VTA neurons. The next step in our study was to investigate the effects of two substances that promote arousal – the neurotransmitter histamine and a novel wake-promoting compound modafinil, which is used for the treatment of narcolepsy.

16.8. Effects of histamine on DAergic and GABAergic neurons in SN and VTA.

This part of study was carried out on drug-naive neurons. Using extracellular recordings of the spontaneous activity of DA neurons in SN we found that bath application of HA (10 μM) did not affect their firing frequency (n=6, **Fig. 16A**). In contrast, bath application of histamine (10 μM) increased the firing of GABAergic neurons in SN pars reticulata to 119,4 ± 4,9 % of control (n=6, $p<0.005$, **Fig. 16 B**)(Korotkova et al., 2002b). Hundred μM of histamine caused a somewhat stronger excitation – to 141,0 ± 14,6% (n=8, **Fig.16 C**), however, this difference was not statistically significant. The effect of histamine (10 μM) was blocked by prior application of the selective H_1 receptor antagonist mepyramine (1 μM, n=6, $p<0.005$). In the presence of the H_2 receptor antagonist cimetidine (50 μM) the response of GABAergic neurons to histamine (10μM) was not blocked (n = 4).

In the VTA, bath application of histamine (100 μM) did not affect the firing of dopamine cells (n = 7), but increased the firing of GABAergic neurons to 119,5 ± 7,8 % of control rate (n = 6, $p<0.01$). Application of a lower dose of histamine (10 μM) caused the same level of excitation of GABAergic neurons (118,5 ± 7,2%, n = 6, $p<0.01$, **Fig. 16D**). This

histamine-induced excitation was blocked by the H_1 receptor antagonist mepyramine (1 µM, n = 4, $p<0.05$, **Fig. 16E**). In the presence of the H_2 receptor antagonist cimetidine (50 µM, n = 3, **Fig.16 F**) or H_3 receptor antagonist thioperamide (10 µM, n = 4, **Fig. 16G**) the response of GABAergic neurons to histamine was not blocked. These results are summarized in Table 10.

We also examined if inhibition of DAergic neurons by GABAergic neurons takes place in vitro. Application of the $GABA_A$ receptor antagonist bicuculline (20 µM, n=7) or the $GABA_B$ antagonist CGP55845 (100 nM, n=3) failed to change the firing rate of DAergic neurons in both regions, suggesting that input from GABAergic neurons remaining in the slice after cutting is not sufficient to change the firing rate of DAergic neurons.

Table 10. Effects of histamine on the SN and VTA cells.

Cell subtype	N of exper.	hist.receptors antagonists	concentration	Histam.(conc.)	Eff (P value)
SN DA	6			10µM	no effect
SN GABA	6			10µM	119,4 ± 4,9% p<0.005
SN GABA	8			100µM	141,0± 14,6% p<0.0001
SN GABA	6	mepyramine	1µM	10µM	inhibition of resp.,p<0.005
SN GABA	4	cimetidine	50 µM	10µM	no inhibition of response
VTA DA	7			100µM	no effect
VTA GABA	6			100µM	119,5 ± 7,8 % p<0.01
VTA GABA	6			10µM	118,5 ± 7,2% p<0.01
VTA GABA	4	mepyramine	1µM	10µM	inhibition of resp.,p<0.05
VTA GABA	3	cimetidine	50 µM	10µM	no inhibition of response
VTA GABA	4	thioperamide	10µM	10µM	no inhibition of response

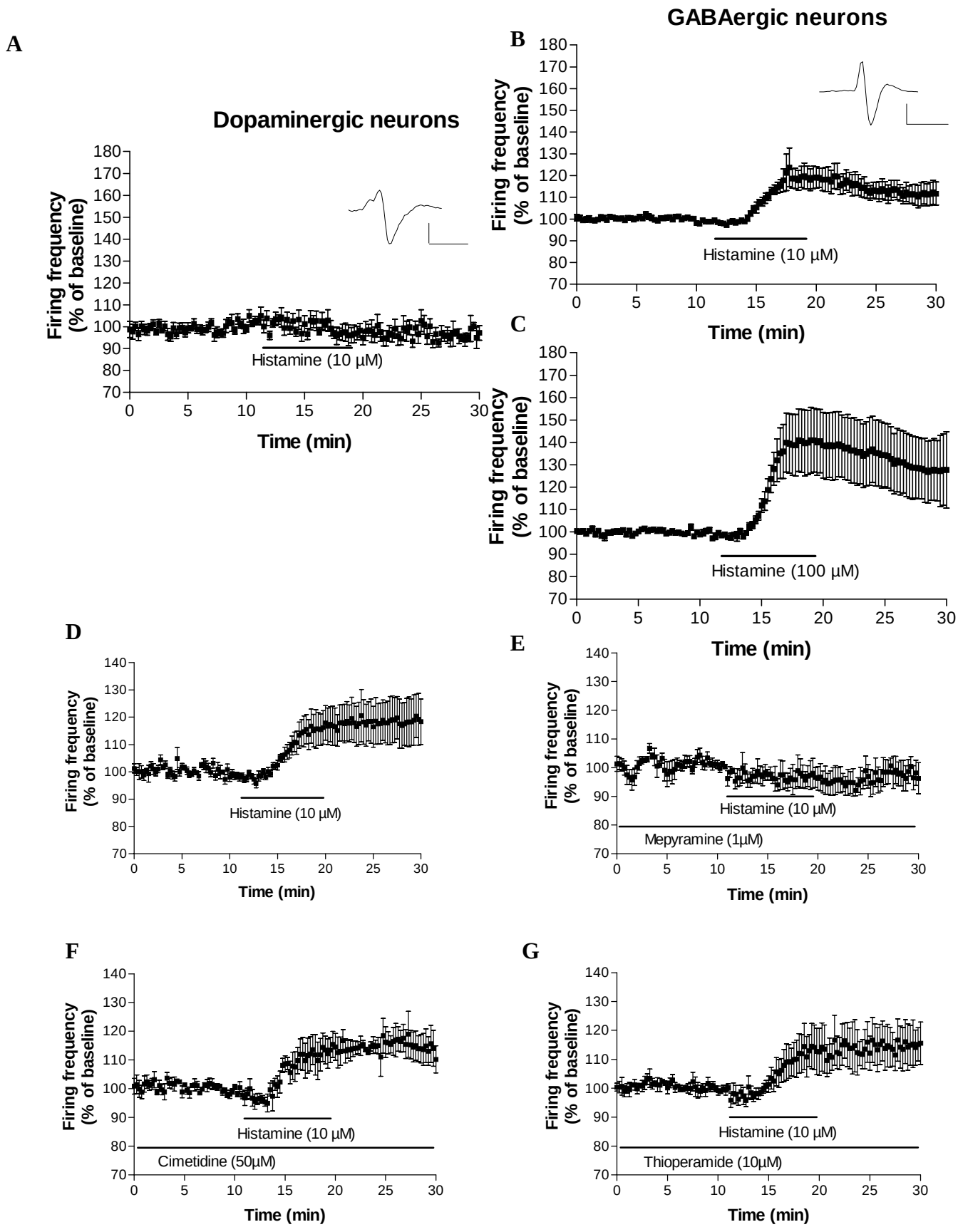

A
Dopaminergic neurons
Firing frequency (% of baseline)
Histamine (10 µM)
Time (min)
B
GABAergic neurons
Firing frequency (% of baseline)
Histamine (10 µM)
Time (min)
C
Firing frequency (% of baseline)
Histamine (100 µM)
Time (min)
D
Firing frequency (% of baseline)
Histamine (10 µM)
Time (min)
E
Firing frequency (% of baseline)
Histamine (10 µM)
Mepyramine (1µM)
Time (min)
F
Firing frequency (% of baseline)
Histamine (10 µM)
Cimetidine (50µM)
Time (min)
G
Firing frequency (% of baseline)
Histamine (10 µM)
Thioperamide (10µM)
Time (min)

Figure 16. Influence of histamine on the firing rate of neurons in the SN and VTA.

A. Histamine (10 μM) did not affect the firing rate of DAergic neurons in SN.

B,**C.** Histamine-induced increase of GABAergic neuron firing in SN.

Inset: **A**, **B** –single action potentials

recorded from DAergic and GABAergic neurons, respectively. Each trace is the average of 15 individual recordings. Calibration bars vertical: 1 ms; horizontal: 0.5mV.

D. Histamine (10 μM) increased the firing frequency of GABAergic neurons in the ventral tegmental area.

E.

Prior bath application of H_1 receptor antagonist mepyramine (1 μM) blocked the histamine-induced excitation.

F,G. H_2 receptor antagonist cimetidine (50 μM) and H_3 receptor antagonist thioperamide (10 μM) had no influence on the effect of histamine.

16.9. Action of modafinil on SN and VTA neurons.

This study was made on drug-naive neurons. It is still in progress but I would like to present the preliminary findings. As amphetamines inhibit DAergic neurons in both SN and VTA in vitro and the authors who investigate action of psychostimulants, often refer to both midbrain DAergic nuclei as to an indistinguishable group (Wisor et al., 2001), we pooled data from both nuclei together for the preliminary study. Bath application of modafinil (20μM) inhibited DAergic neurons to 61.0 ± 6.0% of control both in VTA and in SN (n=7, $p<0.001$, **Fig.17 A, B**). The prior application of the voltage-gated sodium channel blocker tetrodotoxin (0.5 μM) did not prevent the hyperpolarization caused by modafinil, indicating a direct postsynaptic effect (n=1, **Fig.17C**). We are going to investigate the involvement of dopamine receptors, dopamine reuptake transporter and adrenergic receptors in the action of modafinil on the midbrain DAergic system.

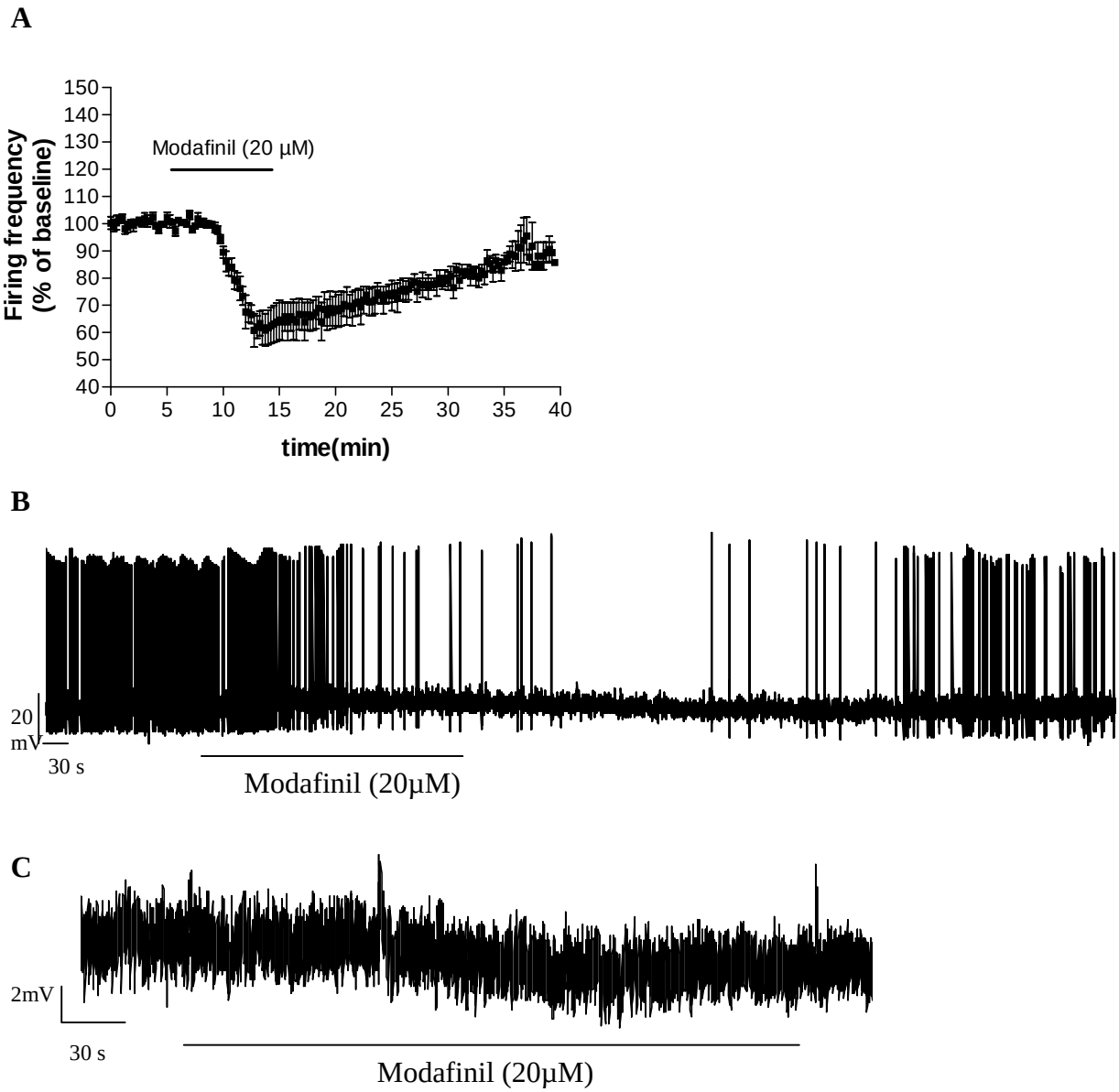


Figure 17. Action of modafinil on DAergic neurons in SN and VTA.
A, B.Bath application of modafinil (20µM) inhibited DAergic neurons to 61.0±6.0% of control both in VTA and in SN.
C. The prior application of tetrodotoxin (0.5 µM) did not prevent the modafinil-induced hyperpolarization

17. Discussion

We found that in the substantia nigra pars compacta dopamine neuronswere unaffected by bath applied orexin A, which activates both types of orexin receptors with high affinity (Sakurai et al., 1998), whereas GABAergic cells in the pars reticulata were strongly excited by both orexin A and orexin B. These results were unexpected because a previous study of the distribution of orexin fibres reported a complete absence of orexin-containing fibres from the substantia nigra pars reticulata (Peyron et al., 1998). We have re-examined this question using immunohistochemical methods and confirm that orexin fibres are almost completely absent in the rostral substantia nigra, their density steadily increases in the rostral-caudal direction. Thus, the anatomical substrate for an orexin modulation of these cells exists. The second reason why these results were unexpected was the fact that other aminergic neurons are all potently excited by orexins. However, it is notable that whereas the firing of the other aminergic neurons mentioned varies across the sleep-wake cycle, that of the dopamine neurons does not (Miller et al., 1983) and so the lack of modulation by orexins is entirely consistent with their role in arousal. A previous study found that orexin A-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system (Nakamura et al., 2000). Our results suggest that these effects are not mediated by orexin actions on nigrostriatal dopamine neurons but rather on mesocorticolimbic dopamine neurons located in the ventral tegmental area (Korotkova et al., 2003), which exhibit an increase in intracellular calcium (Nakamura et al., 2000) and increased firing in response to orexin A.

In contrast to the SN dopamine neurons the firing rate of GABAergic cells in the pars reticulata is sensitive to behavioural state (Miller et al., 1983). These cells fire faster during waking and during rapid-eye movement (REM) sleep in comparison with slow-wave sleep. We found in vitro that these cells had a firing rate of 8.6 ± 0.8 Hz, which was increased to 16.6 ± 0.8 Hz by 100 nM Orexin B. During the waking state in vivo these cells fire at 12-20 Hz (Miller et al., 1983). Since orexin neurons are most active during the waking state (Estabrooke et al., 2001;Taheri et al., 2000), orexins (together with biogenic amines – see below) may act to maintain the high firing rate of these cells during waking.

The response to orexins in GABAergic pars reticulata cells was very homogeneous. Thus, we were able to investigate the pharmacology and signal transduction mechanisms of this excitation in detail. Orexin A and B were similarly efficacious in exciting these cells at 100 nM and the dose-response curve for orexin B had an EC_{50} in the low nanomolar range (12 nM). These observations suggest that the type II orexin receptor is involved in mediating

these effects since in transfected CHO cells orexin A and B are similarly potent at the type II receptor (EC_{50}s 34 and 60 nM respectively) whereas orexin B has an EC_{50} in the micromolar range (2.5 µM) for activating the type I receptor (Sakurai et al., 1998). Although Marcus et al., 2001, did not find expression of mRNA for the orexin type II or type I receptor in the substantia nigra pars reticulata, these findings depend on the sensitivity of the in situ histochemistry and may represent false negatives. The type II receptor seems to be more important in the control of the sleep-wake cycle and in the context of narcolepsy, since disruption of the OX_2R receptor gene leads to narcolepsy (Willie et al., 2001), whereas disruption of the OX_1R gene alone does not lead to obvious behavioural abnormalities (such as cataplectic attacks) and these animals only exhibit fragmentation of the sleep-wake cycle. Consequently, the orexin action on reticulata cells is likely to be related to regulation of activity across the sleep-wake cycle.

It has been reported that orexin receptors are coupled to phospholipase C (Sakurai et al., 1998;van den Pol et al., 1998). This enzyme generates two second messengers – inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium from internal stores whereas DAG potentiates the activity of PKC. Accordingly, we tested inhibitors of these pathways for their ability to block the orexin B induced excitation of reticulata cells. Application of thapsigargin reduced the effect of orexin B but did not abolish it, indicating that calcium release from internal stores plays a role in the excitation of these cells. In contrast, influx of extracellular calcium does not seem to be necessary, since the magnitude of the orexin B effect was unchanged in low calcium/high magnesium solution. Application of a high concentration of the broad-spectrum kinase inhibitor H7 also reduced the magnitude of the orexin B effect, but, surprisingly, the selective protein kinase C inhibitor chelerythrine was ineffective. Instead, two protein kinase inhibitors (H89 and KT5720) applied at concentrations which selectively block protein kinase A were able to block the orexin B-induced excitation. Coadministration of H7 and thapsigargin did not reduce the magnitude of the orexin B effect to a greater extent than either drug alone, suggesting that the release of calcium from internal stores and the activation of protein kinase A are not independent of each other – one possibility is that calcium released from internal stores activates calcium-sensitive adenylyl cyclase in these cells.

The firing of GABAergic reticulata cells is extremely important for the regulation of muscle tone. Abnormal increases in the firing of these cells seen in Parkinson`s disease or following neuroleptic treatement lead to catalepsy (Niijima and Yoshida, 1982; Blandini et al., 2000;Boulay et al., 2000;Evans et al., 2001). We propose that an impaired orexin system,

as seen in narcolepsy, leads to an abnormally low activity in these cells and predisposes individuals to what is, in terms of muscle tone, the reverse effect, namely cataplexy. Both direct and indirect mechanisms are likely to play a role. Thus, we have shown here that orexins directly and potently excite these cells. In addition, though, orexins excite serotonergic (Brown et al., 2001a), histaminergic (Eriksson et al., 2001) and noradrenergic neurons (Hagan et al., 1999) and noradrenaline (Berretta et al., 2000), serotonin (Stanford and Lacey, 1996) and histamine(see the following part of the study) in turn excite GABAergic reticulata cells. Thus, in narcoleptics the action of these neurotransmitters is also likely to be reduced. Recently, it has been shown that oral application of sulpiride, a dopamine D_2-like receptor antagonist, which will indirectly increase the firing of reticulata cells, effectively blocks cataplexy without affecting REM sleep (Okura et al., 2000). To summarize, the presence of an abnormally low basal activity of substantia nigra pars reticulata cells may allow the expression of cataplexy, which is prevented in normal individuals by the inhibitory projections of reticulata cells to the ventromedial thalamus and medial pontine reticular formation (Niijima and Yoshida, 1982). This hypothesis can be tested experimentally by recording from these cells in narcoleptic animals.

GABAergic cells in the VTA are also excited by orexins. We found two groups of GABAergic neurons in the VTA - fast-firing and slow-firing. The existence of the first group is well known, but the latter one has not yet been described. We did not find slow-firing GABAergic cells in SN. The further study of the projection pattern of these cells and their neurochemical and electrophysiological properties could help to understand the other differences of these groups in VTA. The depolarization of both groups of GABAergic cells persisted in the presence of tetrodotoxin indicating that this was also a direct action of orexins. Similar to our findings in the substantia nigra, the effect of orexins on firing rate in fast-firing cells of the pars reticulata was blocked by inhibition of protein kinase A. In recent years it has become clear, that the contribution of GABAergic neurons in the reward processes is more complicated than just inhibition of DAergic neurons. Steffensen et al (Steffensen et al., 2001) have shown that the spontaneous activity of GABAergic neurons in VTA increased progressively, reaching a peak around 1–2 s before the time of an active intracranial self-stimulation. Such an increase in activity of GABAergic VTA neurons during the approach may reflect the rat's attention to the expected rewarding stimulus. This indicates the involvement of the orexin system in reward processes. This hypothesis is further supported by the finding that orexins excite the majority of neurons in the ventral tegmental area of the rat. While GABAergic cells were uniformly excited, dopaminergic cells showed a

variety of responses. One group of cells was unaffected by orexins, similar to our findings with dopaminergic neurons in the substantia nigra. A second group of cells showed a large increase in firing frequency, which was associated with a depolarization. The depolarizing effect of orexins was not blocked by the voltage-gated sodium channel blocker tetrodotoxin andtherefore likely represents a direct effect on the recorded neurons. The third group of dopaminergic cells, which we encountered had their firing pattern changed by the application of orexins to periods with increased firing separated by silent periods. In intracellular recordings, silent periods were either associated with hyperpolarizations or with large depolarizations leading to inactivation of voltage-gated sodium channels. In several cells burst firing was observed – burst firing is commonly observed in vivo and is associated with the unexpected appearance of rewards or stimuli predicting reward (Schultz, 1998). In vitro, a burst firing pattern, somewhat different from "natural" bursts, can be elicited by application of nickel, alone or in combination with apamin, which blocks a slow afterhyperpolarization (Wolfart and Roeper, 2002), or by NMDA together with apamin, which blocks a slow afterhyperpolarization (Seutin et al., 1993). In that regard it is interesting that those cells, which later showed oscillatory responses to orexins had a significantly reduced AHP in contrast to other dopaminergic cells. What other differences could be found in these neurons? We performed single-cell RT-PCR to study mRNA expression in a given neuron. We found that both orexin receptors were expressed in VTA cells. Many cells contained only the OX_1 receptor, which is consistent with our finding that occasional cells responded to orexin A but not to orexin B in slice experiments. Calbindin (CB) was expressed in half of the VTA cells that expressed orexin receptors but was never detected in cells that lacked orexin receptors. In mice the TH^+/CB^+ cells had smaller afterhyperpolarizations than TH^+/CB^- VTA neurons (Neuhoff et al., 2002); so this group of cells may correspond to the cells in which we found oscillatory responses to orexins. A subpopulation of TH^+ cells was also positive for GAD65 – recently the existence of such a group of cells has been demonstrated using anatomical techniques (Gonzalez-Hernandez et al., 2001;Carr and Sesack, 2000). They are likely to be excited by orexins, since we have shown that they expressed orexin receptors. All TH^+/GAD^+ neurons were CB-negative.

In dorsal raphe, where neurons are also excited by orexins (Brown et al., 2001a) the expression of orexin receptors is correlated with the expression of transient receptor potential TRPC6- and TRPC5- channel subunits (Sergeeva et al., 2003). However, in VTA and other aminergic nuclei (tuberomamillary nucleus, locus coeruleus) the expression of orexin receptors did not correlate with the occurrence of TRPC channels. Thus, TRPC channels may

not represent a general effector mechanism of orexin receptor action. The cell-type specific regulation of receptor channel coupling needs to be elucidated in future studies.

Dopaminergic and GABAergic neurons in the VTA were excited by orexin A and orexin B and possess both types of orexin receptors, suggesting that they play a role in both the arousal/narcolepsy and feeding aspects of the function of orexins. Cataplexy is elicited in narcoleptics by emotional arousal (Nishino and Mignot, 1997). In narcoleptic dogs, which have a dysfunctional orexin type II receptor, the most commonly used assay for cataplexy is the food-elicited cataplexy test (Nishino and Mignot, 1997). Given the role played by VTA dopamine neurons in the response to primary rewards such as food, dysfunction of the orexin-regulation of dopamine neurons is likely to be an important component of the triggering mechanism for cataplexy (Reid et al., 1996). In normal individuals, the orexin modulation of VTA neurons is likely to be important in transmitting information about the availability of primary rewards to the mesocorticolimbic reward system. A subpopulation of orexin neurons possess leptin receptors (Willie et al., 2001;Hakansson et al., 1999) and leptin administration is known to modulate the rewarding effect of lateral hypothalamic stimulation (Fulton et al., 2000). Furthermore, orexin neurons are sensitive to metabolic state, receiving input from glucose-sensitive neurons (Liu et al., 2001). One model of the role of the orexin modulation of VTA neurons could be as follows: lack of adequate metabolic substrate would lead to increased activity in orexin neurons, excitation of dopaminergic and GABAergic neurons in the VTA, leading to increased arousal, locomotor activity and a search for food.

Melanin-concentrating hormone is located in neurons intermingled with the orexin neurons in the perifornical area. Melanin concentrating hormone (MCH) containing fibres are present in the SN and VTA (Bittencourt et al., 1992), as is the receptor for MCH, SLC-1 (Chen and Yau, 1994). However, MCH did not affect the firing of any of the neurons we investigated when tested at similar concentrations to the orexins, suggesting that it may primarily act on synaptic transmission, rather than directly on cellular excitability. Recently it has indeed been shown that MCH inhibits synaptic transmission in cultures of lateral hypothalamic neurons (Gao and van den Pol, 2002). MCH could exert its effects in nucleus accumbens – it contains very high levels of MCH receptor mRNA expression and MCH ligand binding sites (Borowsky et al., 2002). We found that orexins did not affect field potentials in nucleus accumbens and dorsal striatum. A more detailed study (such as whole-cell patch-clamp recordings of striatal neurons) should reveal any action of orexins in the striatum.

The next aim of the study was to investigate if other hypothalamic peptides also affect VTA neurons or if this is a unique property of the orexins. Neuropeptide Y (NPY), another potent food intake stimulator, inhibited nearly a half of the dopaminergic and GABAergic neurons tested. The anorectic hypothalamic neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH) had no effect on DAergic cells. Corticotropin-releasing factor (CRF), an anorectic peptide, which is also known to promote arousal, excited 60% of dopaminergic and all tested GABAergic neurons. Neither the orexigenic neuropeptides ghrelin, melanin-concentrating hormone (MCH) and agouti-related protein (AGRP) nor the anorectic peptides leptin and cocaine and amphetamine-related transcript (CART) affected the firing rate or membrane potential of VTA neurons. Substance P (SP) that promotes arousal but not food intake, excited the majority of DAergic VTA neurons and all tested GABAergic neurons. These diverse pilot data suggest that there is no clear relationship between the ability of hypothalamic peptides to stimulate food intake and their ability to excite or inhibit VTA neurons. In contrast, the association with the action of peptide on arousal, is more clear: peptides which increase arousal all seem to excite VTA neurons and NPY which is known to possess anxiolytic properties, inhibits VTA neurons. Except for the preliminary finding of opposite effect of alpha-MSH and NPY on the same GABAergic neurons, which has to be studied further, no relationship in actions of other tested peptides on the same DAergic or GABAergic neurons was found, suggesting the mediation of effects through the different VTA neurons rather than a counter-regulation of these peptides via reciprocal effects on the same cells. This observation was confirmed by RT-PCR study, which did not yet reveal any link in expression of orexin receptors, NPY, CART, SP and MC4 receptors in VTA neurons. Interestingly, in most cases the given peptide affected DAergic and GABAergic neurons in the same, but not the opposite, way – excited or inhibited both types simultaneously. It's possible to suggest that it could be necessary for an excitation of selected dopaminergic pathways and an inhibition of another group of dopaminergic neurons (through excitation of GABAergic neurons), so that only particular signal could reach projection targets.

As far as arousal-promoting hypothalamic peptides seemed to affect the VTA cells in a similar way, in direction opposite to the classical arousal-promoting compound amphetamine, which inhibits DAergic neurons (Bunney et al., 1973), we studied the action of two other strong wake-promoting substances: the neuromodulator histamine and a novel compound modafinil. We have found that both in the SN and the VTA, histamine does not affect the spontaneous firing of DA neurons in pars compacta, but causes an excitation of GABAergic

neurons in SN pars reticulata. This excitation could also cause an inhibition of DAergic neurons SN pars compacta in vivo (Paladini et al., 1999), therefore leading to inhibition of nigrostriatal dopaminergic transmission, that might explain the histamine-induced initial decrease in locomotory activity (Chiavegatto et al., 1998). As we have shown, this inhibition does not take place in vitro – application of $GABA_A$ or $GABA_B$ antagonists did not change the firing rate of DAergic neurons, suggesting that the majority of GABAergic axons was cut during preparation of slices and that the remaining inputs were not strong enough to influence the activity of DAergic neurons.

In the VTA, histamine also does not affect dopaminergic neurons, but may inhibit them in vivo through excitation of GABAergic neurons, thus explaining the functional antagonism between the histaminergic and dopaminergic systems, desribed above (Huston et al., 1997).

In the context of the involvement of the histamine system in arousal mechanisms and the circadian rhythm of histamine release in brain, it is noteworthy that HA did not change the activity of DAergic neurons, whose firing rate does not vary with the sleep-wake cycle, but increased the firing rate of GABAergic neurons that are known to fire more during arousal compared to REM sleep and during REM sleep compared to slow-wave sleep (Miller et al., 1983).

In the last part of our study we found that modafinil, similar to amphetamine (Bunney et al., 1973) and cocaine (Bunney et al., 2001), inhibited DAergic neurons in VTA and SN in vitro. Although in an in vivo study no effect of modafinil on VTA neurons was found (Akaoka et al., 1991), it could be explained by the low solubility of modafinil (it may have precipitated before reaching the target neuron) and the small number of experiments in that study. Together with the findings that modafinil exhibits a weak affinity for the dopamine transporter (DAT) (Mignot et al., 1994) and the wake-promoting effects of classical stimulants and modafinil are abolished in DAT knock-out mice (Wisor et al., 2001), our data confirm the hypothesis that effects of modafinil are mediated, at least partly, through the dopaminergic system.

18. Summary

1. Orexins uniformly excited GABAergic neurons in SN and VTA.

2. Orexin-mediated excitation of GABAergic neurons in SN and VTA was blocked by the prior application of a selective protein kinase A inhibitor.

3. A distinct subgroup of GABAergic neurons in VTA with a slow firing rate (0.8 Hz) was found.

4. Orexins caused an increase in firing frequency (EC_{50} 78nM), burst firing or no change in firing in different groups of A10 dopamine neurons.

5. Neurons showing oscillatory firing in response to orexins had smaller afterhyperpolarizations than the other groups of dopamine neurons.

6. Single-cell RT-PCR experiments showed that orexin receptors were expressed in both dopaminergic and non-dopaminergic neurons and that the calcium binding protein calbindin was only expressed in neurons, which also expressed orexin receptors.

7. A group of VTA neurons, which express both TH and GAD, was also investigated by RT-PCR. All such neurons expressed orexin receptors and did not express calbindin. The soma size of these neurons was similar to TH-positive GAD-negative neurons and was significantly larger than in TH- negative GAD-positive neurons.

8. The following relationships between soma size and cell properties were found: CB^+ /TH^+ positive cells had a significantly smaller soma size than CB^-/TH^+ negative cells. TH-negative neurons, which expressed at least 1 type of orexin receptor were significantly smaller than TH-negative cells that did not express orexin receptors.

9. In VTA, in contrast to dorsal raphe, the expression of the two orexin receptors was not related with the presence or absence of TRPC subunits.

10. Orexins did not affect field potentials in ventral and dorsal striatum.

11. Melanin-concentrating hormone (MCH), agouti-related protein (AGRP), ghrelin, leptin and cocaine and amphetamine-related transcript (CART) did not affect membrane potential or firing rate of the VTA neurons.

12. Neuropeptide Y (NPY) inhibited 40% of DAergic cells and 55.6% of fast-firing GABAergic neurons in VTA.

13. α-Melanocyte stimulating hormone (α-MSH) had no effect on firing rate or membrane potential of DAergic cells.

14. Corticotropin-releasing factor (CRF) excited a subgroup of DAergic neurons and all tested GABAergic neurons as well.

15. Substance P (SP) increased the firing rate of the majority of DAergic and all tested GABAergic neurons in VTA.

16. Histamine did not affect the firing frequency of DAergic neurons but increased the firing of GABAergic neurons in SN and VTA. This effect was blocked by prior application of the selective H_1 receptor antagonist mepyramine.

17. Modafinil inhibited DAergic neurons both in VTA and SN.

19. Reference List

Aghajanian GK, Rasmussen K (1989) Intracellular studies in the facial nucleus illustrating a simple new method for obtaining viable motoneurons in adult rat brain slices. Synapse 3: 331-338.

Airaksinen MS, Thoenen H, Meyer M (1997) Vulnerability of midbrain dopaminergic neurons in calbindin-D28k- deficient mice: lack of evidence for a neuroprotective role of endogenous calbindin in MPTP-treated and weaver mice. Eur J Neurosci 9: 120-127.

Akaoka H, Roussel B, Lin JS, Chouvet G, Jouvet M (1991) Effect of modafinil and amphetamine on the rat catecholaminergic neuron activity. Neurosci Lett 123: 20-22.

Albanese A, Minciacchi D (1983) Organization of the ascending projections from the ventral tegmental area: a multiple fluorescent retrograde tracer study in the rat. J Comp Neurol 216: 406-420.

Alvaro JD, Tatro JB, Duman RS (1997) Melanocortins and opiate addiction. Life Sci 61: 1-9.

Anand BK, Brobeck JR (1951) Localization of a "feeding center" in the hypothalamus of the rat. Proc Soc Exp Biol Med ***77***: 323-324.

Aosaki T, Tsubokawa H, Ishida A, Watanabe K, Graybiel AM, Kimura M (1994) Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning. J Neurosci 14: 3969-3984.

Barrot M, Calza L, Pozza M, Le Moal M, Piazza PV (2000) Differential calbindin-immunoreactivity in dopamine neurons projecting to the rat striatal complex. Eur J Neurosci 12: 4578-4582.

Bayer VE, Pickel VM (1991) GABA-labeled terminals form proportionally more synapses with dopaminergic neurons containing low densities of tyrosine hydroxylase-immunoreactivity in rat ventral tegmental area. Brain Res 559: 44-55.

Beal MF (2000) Energetics in the pathogenesis of neurodegenerative diseases. Trends Neurosci 23: 298-304.

Beart PM, McDonald D (1980) Neurochemical studies of the mesolimbic dopaminergic pathway: somatodendritic mechanisms and GABAergic neurones in the rat ventral tegmentum. J Neurochem 34: 1622-1629.

Beck B, Richy S, Dimitrov T, Stricker-Krongrad A (2001) Opposite regulation of hypothalamic orexin and neuropeptide Y receptors and peptide expressions in obese Zucker rats. Biochem Biophys Res Commun 286: 518-523.

Beckstead RM, Domesick VB, Nauta WJ (1979) Efferent connections of the substantia nigra and ventral tegmental area in the rat. Brain Res 175: 191-217.

Berretta N, Bernardi G, Mercuri NB (2000) Alpha(1)-adrenoceptor-mediated excitation of substantia nigra pars reticulata neurons. Neuroscience 98: 599-604.

Berridge KC, Robinson TE (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res Brain Res Rev 28: 309-369.

Bester H, Besson JM, Bernard JF (1997) Organization of efferent projections from the parabrachial area to the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. J Comp Neurol 383: 245-281.

Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, Vale W, Sawchenko PE (1992) The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J Comp Neurol 319: 218-245.

Blandini F, Nappi G, Tassorelli C, Martignoni E (2000) Functional changes of the basal ganglia circuitry in Parkinson's disease. Prog Neurobiol 62: 63-88.

Borowsky B, Durkin MM, Ogozalek K, Marzabadi MR, DeLeon J, Lagu B, Heurich R, Lichtblau H, Shaposhnik Z, Daniewska I, Blackburn TP, Branchek TA, Gerald C, Vaysse PJ, Forray C (2002) Antidepressant, anxiolytic and anorectic effects of a melanin- concentrating hormone-1 receptor antagonist. Nat Med 8: 825-830.

Boulay D, Depoortere R, Oblin A, Sanger DJ, Schoemaker H, Perrault G (2000) Haloperidol-induced catalepsy is absent in dopamine D(2), but maintained in dopamine D(3) receptor knock-out mice. Eur J Pharmacol 391: 63-73.

Broberger C, de Lecea L, Sutcliffe JG, Hokfelt T (1998) Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. J Comp Neurol 402: 460-474.

Brodie MS, McElvain MA, Bunney EB, Appel SB (1999) Pharmacological reduction of small conductance calcium-activated potassium current (SK) potentiates the excitatory effect of ethanol on ventral tegmental area dopamine neurons. J Pharmacol Exp Ther 290: 325-333.

Brown RE, Sergeeva O, Eriksson KS, Haas HL (2001a) Orexin A excites serotonergic neurons in the dorsal raphe nucleus of the rat. Neuropharmacology 40: 457-459.

Brown RE, Sergeeva OA, Eriksson KS, Haas HL (2002) Convergent excitation of dorsal raphe serotonin neurons by multiple arousal systems (orexin/hypocretin, histamine and noradrenaline). J Neurosci 22: 8850-8859.

Brown RE, Stevens DR, Haas HL (2001b) The physiology of brain histamine. Prog Neurobiol 63: 637-672.

Bunney BS, Walters JR, Roth RH, Aghajanian GK (1973) Dopaminergic neurons: effect of antipsychotic drugs and amphetamine on single cell activity. J Pharmacol Exp Ther 185: 560-571.

Bunney EB, Appel SB, Brodie MS (2001) Electrophysiological effects of cocaethylene, cocaine, and ethanol on dopaminergic neurons of the ventral tegmental area. J Pharmacol Exp Ther 297: 696-703.

Cador M, Kelley AE, Le Moal M, Stinus L (1986a) Ventral tegmental area infusion of substance P, neurotensin and enkephalin: differential effects on feeding behavior. Neuroscience 18: 659-669.

Campbell KJ, Takada M, Hattori T (1991) Co-localization of tyrosine hydroxylase and glutamate decarboxylase in a subpopulation of single nigrotectal projection neurons. Brain Res 558: 239-244.

Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P (1995) Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269: 546-549.

Cardozo DL, Bean BP (1995) Voltage-dependent calcium channels in rat midbrain dopamine neurons: modulation by dopamine and GABAB receptors. J Neurophysiol 74: 1137-1148.

Carr DB, Sesack SR (2000) GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. Synapse 38: 114-123.

Carr KD (1996) Feeding, drug abuse, and the sensitization of reward by metabolic need. Neurochem Res 21: 1455-1467.

Carr KD, Wolinsky TD (1993) Chronic food restriction and weight loss produce opioid facilitation of perifornical hypothalamic self-stimulation. Brain Res 607: 141-148.

Cauli B, Audinat E, Lambolez B, Angulo MC, Ropert N, Tsuzuki K, Hestrin S, Rossier J (1997) Molecular and physiological diversity of cortical nonpyramidal cells. J Neurosci 17: 3894-3906.

Charlety PJ, Grenhoff J, Chergui K, de La CB, Buda M, Svensson TH, Chouvet G (1991) Burst firing of mesencephalic dopamine neurons is inhibited by somatodendritic application of kynurenate. Acta Physiol Scand 142: 105-112.

Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98: 437-451.

Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP (1996) Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell 84: 491-495.

Chen TY, Yau KW (1994) Direct modulation by Ca(2+)-calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. Nature 368: 545-548.

Chergui K, Suaud-Chagny MF, Gonon F (1994) Nonlinear relationship between impulse flow, dopamine release and dopamine elimination in the rat brain in vivo. Neuroscience 62: 641-645.

Chiavegatto S, Nasello AG, Bernardi MM (1998) Histamine and spontaneous motor activity: biphasic changes, receptors involved and participation of the striatal dopamine system. Life Sci 62: 1875-1888.

Chiodo LA, Bannon MJ, Grace AA, Roth RH, Bunney BS (1984b) Evidence for the absence of impulse-regulating somatodendritic and synthesis-modulating nerve terminal autoreceptors on subpopulations of mesocortical dopamine neurons. Neuroscience 12: 1-16.

Cohn CK, Ball GG, Hirsch J (1973) Histamine: effect on self-stimulation. Science 180: 757-758.

Contarino A, Heinrichs SC, Gold LH (1999) Understanding corticotropin releasing factor neurobiology: contributions from mutant mice. Neuropeptides 33(1): 1-12.

Cote NK, Harrington ME (1993) Histamine phase shifts the circadian clock in a manner similar to light. Brain Res 613: 149-151.

Cowley MA, Pronchuk N, Fan W, Dinulescu DM, Colmers WF, Cone RD (1999) Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat. Neuron 24: 155-163.

Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411: 480-484.

Cummings DE, Clement K, Purnell JQ, Vaisse C, Foster KE, Frayo RS, Schwartz MW, Basdevant A, Weigle DS (2002) Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med 8: 643-644.

Dallvechia-Adams S, Kuhar MJ, Smith Y (2002) Cocaine- and amphetamine-regulated transcript peptide projections in the ventral midbrain: Colocalization with -aminobutyric acid, melanin-concentrating hormone, dynorphin, and synaptic interactions with dopamine neurons. J Comp Neurol 448: 360-372.

David V, Durkin TP, Cazala P (1997) Self-administration of the GABAA antagonist bicuculline into the ventral tegmental area in mice: dependence on D2 dopaminergic mechanisms. Psychopharmacology (Berl) 130: 85-90.

de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 95: 322-327.

Deniau JM, Menetrey A, Charpier S (1996) The lamellar organization of the rat substantia nigra pars reticulata: segregated patterns of striatal afferents and relationship to the topography of corticostriatal projections. Neuroscience 73: 761-781.

Denner LA, Wu JY (1985) Two forms of rat brain glutamic acid decarboxylase differ in their dependence on free pyridoxal phosphate. J Neurochem 44: 957-965.

Di MV, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature 410: 822-825.

Doreulee N, Yanovsky Y, Flagmeyer I, Stevens DR, Haas HL, Brown RE (2001) Histamine H(3) receptors depress synaptic transmission in the corticostriatal pathway. Neuropharmacology 40: 106-113.

Douglass J, McKinzie AA, Couceyro P (1995) PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. J Neurosci 15: 2471-2481.

Dringenberg HC, Souza-Silva MA, Schwarting RK, Huston JP (1998) Increased levels of extracellular dopamine in neostriatum and nucleus accumbens after histamine H1 receptor blockade. Naunyn Schmiedebergs Arch Pharmacol 358: 423-429.

Duteil J, Rambert FA, Pessonnier J, Hermant JF, Gombert R, Assous E (1990) Central alpha 1-adrenergic stimulation in relation to the behaviour stimulating effect of modafinil; studies with experimental animals. Eur J Pharmacol 180: 49-58.

Edwards CM, Abusnana S, Sunter D, Murphy KG, Ghatei MA, Bloom SR (1999) The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin. J Endocrinol 160: R7-12.

Elias CF, Saper CB, Maratos-Flier E, Tritos NA, Lee C, Kelly J, Tatro JB, Hoffman GE, Ollmann MM, Barsh GS, Sakurai T, Yanagisawa M, Elmquist JK (1998) Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. J Comp Neurol 402: 442-459.

Elmquist JK, Ahima RS, Elias CF, Flier JS, Saper CB (1998) Leptin activates distinct projections from the dorsomedial and ventromedial hypothalamic nuclei. Proc Natl Acad Sci U S A 95: 741-746.

Ericson H, Blomqvist A, Köhler C (1991) Origin of neuronal inputs to the region of the tuberomammillary nucleus of the rat brain. J Comp Neurol 311: 45-64.

Ericson H, Watanabe T, Köhler C (1987) Morphological analysis of the tuberomammillary nucleus in the rat brain: delineation of subgroups with antibody against L- histidine decarboxylase as a marker. J Comp Neurol 263: 1-24.

Eriksson KS, Sergeeva O, Brown RE, Haas HL (2001) Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. J Neurosci 21: 9273-9279.

Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR (1994) Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. J Neurosci 14: 1834-1855.

Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, Yanagisawa M, Saper CB, Scammell TE (2001) Fos expression in orexin neurons varies with behavioral state. J Neurosci 21: 1656-1662.

Evans NA, Groarke DA, Warrack J, Greenwood CJ, Dodgson K, Milligan G, Wilson S (2001) Visualizing differences in ligand-induced beta-arrestin-GFP interactions and trafficking between three recently characterized G protein-coupled receptors. J Neurochem 77: 476-485.

Fadel J, Deutch AY (2002) Anatomical substrates of orexin-dopamine interactions: lateral hypothalamic projections to the ventral tegmental area. Neuroscience 111: 379-387.

Ferraro L, Tanganelli S, O'Connor WT, Antonelli T, Rambert F, Fuxe K (1996) The vigilance promoting drug modafinil increases dopamine release in the rat nucleus accumbens via the involvement of a local GABAergic mechanism. Eur J Pharmacol 306: 33-39.

Fiorillo CD, Williams JT (1998) Glutamate mediates an inhibitory postsynaptic potential in dopamine neurons. Nature 394: 78-82.

Fleckenstein AE, Lookingland KJ, Moore KE (1993) Activation of mesolimbic dopaminergic neurons following central administration of histamine is mediated by H1 receptors. Naunyn Schmiedebergs Arch Pharmacol 347: 50-54.

Fletcher PJ, Korth KM, Chambers JW (1999) Selective destruction of brain serotonin neurons by 5,7- dihydroxytryptamine increases responding for a conditioned reward. Psychopharmacology (Berl) 147: 291-299.

Franz O, Liss B, Neu A, Roeper J (2000) Single-cell mRNA expression of HCN1 correlates with a fast gating phenotype of hyperpolarization-activated cyclic nucleotide-gated ion channels (Ih) in central neurons. Eur J Neurosci 12: 2685-2693.

Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS (1995) Leptin levels reflect body lipid content in mice: evidence for diet- induced resistance to leptin action. Nat Med 1: 1311-1314.

Freund TF, Bolam JP, Bjorklund A, Stenevi U, Dunnett SB, Powell JF, Smith AD (1985) Efferent synaptic connections of grafted dopaminergic neurons reinnervating the host neostriatum: a tyrosine hydroxylase immunocytochemical study. J Neurosci 5: 603-616.

Fukagawa K, Sakata T, Shiraishi T, Yoshimatsu H, Fujimoto K, Ookuma K, Wada H (1989) Neuronal histamine modulates feeding behavior through H1- receptor in rat hypothalamus. Am J Physiol 256: R605-11.

Fulton S, Woodside B, Shizgal P (2000) Modulation of brain reward circuitry by leptin. Science 287: 125-128.

Galosi R, Lenard L, Knoche A, Haas H, Huston JP, Schwarting RK (2001) Dopaminergic effects of histamine administration in the nucleus accumbens and the impact of H1-receptor blockade. Neuropharmacology 40: 624-633.

Gao XB, van den Pol AN (2002) Melanin-concentrating hormone depresses L-, N-, and P/Q-type voltage- dependent calcium channels in rat lateral hypothalamic neurons. J Physiol 542: 273-286.

Gariano RF, Groves PM (1988) Burst firing induced in midbrain dopamine neurons by stimulation of the medial prefrontal and anterior cingulate cortices. Brain Res 462: 194-198.

Garris PA, Wightman RM (1994) Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. J Neurosci 14: 442-450.

Gehlert DR (1999) Role of hypothalamic neuropeptide Y in feeding and obesity. Neuropeptides 33: 329-338.

Gerashchenko D, Kohls MD, Greco M, Waleh NS, Salin-Pascual R, Kilduff TS, Lappi DA, Shiromani PJ (2001) Hypocretin-2-saporin lesions of the lateral hypothalamus produce narcoleptic-like sleep behavior in the rat. J Neurosci 21: 7273-7283.

Gonzalez-Hernandez T, Barroso-Chinea P, Acevedo A, Salido E, Rodriguez M (2001) Colocalization of tyrosine hydroxylase and GAD65 mRNA in mesostriatal neurons. Eur J Neurosci 13: 57-67.

Grace AA (1988) In vivo and in vitro intracellular recordings from rat midbrain dopamine neurons. Ann N Y Acad Sci 537: 51-76.

Grace AA (1991) Regulation of spontaneous activity and oscillatory spike firing in rat midbrain dopamine neurons recorded in vitro. Synapse 7: 221-234.

Grace AA, Bunney BS (1984) The control of firing pattern in nigral dopamine neurons: single spike firing. J Neurosci 4: 2866-2876.

Grace AA, Bunney BS (1985) Opposing effects of striatonigral feedback pathways on midbrain dopamine cell activity. Brain Res 333: 271-284.

Grace AA, Onn SP (1989) Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. J Neurosci 9: 3463-3481.

Groenewegen HJ, Berendse HW, Haber SN (1993) Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents. Neuroscience 57: 113-142.

Haas HL, Anderson EG, Hosli L (1973) Histamine and metabolites: their effects and interactions with convulsants on brain stem neurones. Brain Res 51: 269-278.

Haas H, Panula P (2003) The role of histamine and the tuberomamillary nucleus in the nervous system. Nat Rev Neurosci 4:121-130

Haas HL, Wolf P (1977) Central actions of histamine: microelectrophoretic studies. Brain Res 122: 269-279.

Haber SN, Fudge JL, McFarland NR (2000) Striatonigrostriatal pathways in primates form an ascending spiral from the shell to the dorsolateral striatum. J Neurosci 20: 2369-2382.

Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S, Benham CD, Taylor SG, Routledge C, Hemmati P, Munton RP, Ashmeade TE, Shah AS, Hatcher JP, Hatcher PD, Jones DN, Smith MI, Piper DC, Hunter AJ, Porter RA, Upton N (1999) Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc Natl Acad Sci U S A 96: 10911-10916.

Hakansson M, de Lecea L, Sutcliffe JG, Yanagisawa M, Meister B (1999) Leptin receptor- and STAT3-immunoreactivities in hypocretin/orexin neurones of the lateral hypothalamus. J Neuroendocrinol 11: 653-663.

Hasenohrl RU, Souza-Silva MA, Nikolaus S, Tomaz C, Brandao ML, Schwarting RK, Huston JP (2000) Substance P and its role in neural mechanisms governing learning, anxiety and functional recovery. Neuropeptides 34: 272-280.

Haynes AC, Jackson B, Overend P, Buckingham RE, Wilson S, Tadayyon M, Arch JR (1999) Effects of single and chronic intracerebroventricular administration of the orexins on feeding in the rat. Peptides 20: 1099-1105.

Hayward MD, Pintar JE, Low MJ (2002) Selective reward deficit in mice lacking beta-endorphin and enkephalin. J Neurosci 22: 8251-8258.

Heinrichs SC, Menzaghi F, Pich EM, Hauger RL, Koob GF (1993) Corticotropin-releasing factor in the paraventricular nucleus modulates feeding induced by neuropeptide Y. Brain Res 611: 18-24.

Heinrichs SC, Richard D (1999) The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. Neuropeptides 33: 350-359.

Heisler LK, Cowley MA, Tecott LH, Fan W, Low MJ, Smart JL, Rubinstein M, Tatro JB, Marcus JN, Holstege H, Lee CE, Cone RD, Elmquist JK (2002) Activation of central melanocortin pathways by fenfluramine. Science 297: 609-611.

Hollerman JR, Schultz W (1998) Dopamine neurons report an error in the temporal prediction of reward during learning. Nat Neurosci 1: 304-309.

Hsu SY, Hsueh AJ (2001) Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. Nat Med 7: 605-611.

Huang CC, Lee EH (1995) Alteration of corticotropin-releasing factor immunoreactivity in MPTP- treated rats. J Neurosci Res 41: 471-480.

Huston JP, Wagner U, Hasenohrl RU (1997) The tuberomammillary nucleus projections in the control of learning, memory and reinforcement processes: evidence for an inhibitory role. Behav Brain Res 83: 97-105.

Hutcheon B, Yarom Y (2000) Resonance, oscillation and the intrinsic frequency preferences of neurons. Trends Neurosci 23: 216-222.

Ikemoto S, Panksepp J (1996) Dissociations between appetitive and consummatory responses by pharmacological manipulations of reward-relevant brain regions. Behav Neurosci 110: 331-345.

Joel D, Weiner I (2000) The connections of the dopaminergic system with the striatum in rats and primates: an analysis with respect to the functional and compartmental organization of the striatum. Neuroscience 96: 451-474.

Jones BE, Harper ST, Halaris AE (1977) Effects of locus coeruleus lesions upon cerebral monoamine content, sleep-wakefulness states and the response to amphetamine in the cat. Brain Res 124: 473-496.

Kang Y, Kitai ST (1993) Calcium spike underlying rhythmic firing in dopaminergic neurons of the rat substantia nigra. Neurosci Res 18: 195-207.

Kask A, Rago L, Wikberg JE, Schioth HB (1998) Evidence for involvement of the melanocortin MC4 receptor in the effects of leptin on food intake and body weight. Eur J Pharmacol 360: 15-19.

Kaufman DL, Houser CR, Tobin AJ (1991) Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem 56: 720-723.

Kawai K, Sugimoto K, Nakashima K, Miura H, Ninomiya Y (2000) Leptin as a modulator of sweet taste sensitivities in mice. Proc Natl Acad Sci U S A 97: 11044-11049.

Kelley AE, Bakshi VP, Haber SN, Steininger TL, Will MJ, Zhang M (2002) Opioid modulation of taste hedonics within the ventral striatum. Physiol Behav 76: 365-377.

Kelley AE, Smith-Roe SL, Holahan MR (1997) Response-reinforcement learning is dependent on N-methyl-D-aspartate receptor activation in the nucleus accumbens core. Proc Natl Acad Sci U S A 94: 12174-12179.

Kilduff TS, de Lecea L (2001) Mapping of the mRNAs for the hypocretin/orexin and melanin- concentrating hormone receptors: networks of overlapping peptide systems. J Comp Neurol 435: 1-5.

Kimmel HL, Gong W, Vechia SD, Hunter RG, Kuhar MJ (2000) Intra-ventral tegmental area injection of rat cocaine and amphetamine- regulated transcript peptide 55-102 induces locomotor activity and promotes conditioned place preference. J Pharmacol Exp Ther 294: 784-792.

Kirouac GJ, Ganguly PK (1995) Topographical organization in the nucleus accumbens of afferents from the basolateral amygdala and efferents to the lateral hypothalamus. Neuroscience 67: 625-630.

Kita T, Kita H, Kitai ST (1986) Electrical membrane properties of rat substantia nigra compacta neurons in an in vitro slice preparation. Brain Res 372: 21-30.

Kiyatkin EA, Rebec GV (1998) Heterogeneity of ventral tegmental area neurons: single-unit recording and iontophoresis in awake, unrestrained rats. Neuroscience 85: 1285-1309.

Klink R, de Kerchove dA, Zoli M, Changeux JP (2001) Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. J Neurosci 21: 1452-1463.

Kochavi D, Davis JD, Smith GP (2001) Corticotropin-releasing factor decreases meal size by decreasing cluster number in Koletsky (LA/N) rats with and without a null mutation of the leptin receptor. Physiol Behav 74: 645-651.

Kohler M, Hirschberg B, Bond CT, Kinzie JM, Marrion NV, Maylie J, Adelman JP (1996) Small-conductance, calcium-activated potassium channels from mammalian brain. Science 273: 1709-1714.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402: 656-660.

Korotkova TM, Eriksson KS, Haas HL, Brown RE (2002a) Selective excitation of GABAergic neurons in the substantia nigra of the rat by orexin/hypocretin in vitro. Regul Pept 104: 83-89.

Korotkova TM, Haas HL, Brown RE (2002b) Histamine excites GABAergic cells in the rat substantia nigra and ventral tegmental area in vitro. Neurosci Lett 320: 133-136.

Korotkova TM, Sergeeva OA, Eriksson KS, Haas HL, Brown RE (2003) Excitation of ventral tegmental area dopaminergic and nondopaminergic neurons by orexins/hypocretins. J Neurosci 23: 7-11.

Köhler C, Swanson L, Haglund L, Wu J-Y (1985) The cytoarchitecture, histochemistry and projections of the tuberomammillary nucleus in the rat. Neuroscience 16: 85-110.

Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393: 72-76.

Kruger, L., Saporta, S., and Swanson, L. W. Photographic Atlas of the rat brain cell and fibre architecture illustrated in three planes with stereotaxic coordinates. 1995. Cambridge University Press.

Kubota Y, Inagaki S, Kito S, Shimada S, Okayama T, Hatanaka H, Pelletier G, Takagi H, Tohyama M (1988) Neuropeptide Y-immunoreactive neurons receive synaptic inputs from dopaminergic axon terminals in the rat neostriatum. Brain Res 458: 389-393.

Kunii K, Yamanaka A, Nambu T, Matsuzaki I, Goto K, Sakurai T (1999) Orexins/hypocretins regulate drinking behaviour. Brain Res 842: 256-261.

Lacey MG, Mercuri NB, North RA (1989) Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. J Neurosci 9: 1233-1241.

Liang CL, Sinton CM, Sonsalla PK, German DC (1996) Midbrain dopaminergic neurons in the mouse that contain calbindin-D28k exhibit reduced vulnerability to MPTP-induced neurodegeneration. Neurodegeneration 5: 313-318.

Lin JS, Roussel B, Akaoka H, Fort P, Debilly G, Jouvet M (1992) Role of catecholamines in the modafinil and amphetamine induced wakefulness, a comparative pharmacological study in the cat. Brain Res 591: 319-326.

Lin JS, Sakai K, Vanni MG, Jouvet M (1989) A critical role of the posterior hypothalamus in the mechanisms of wakefulness determined by microinjection of muscimol in freely moving cats. Brain Res 479: 225-240.

Lindblom J, Opmane B, Mutulis F, Mutule I, Petrovska R, Klusa V, Bergstrom L, Wikberg JE (2001) The MC4 receptor mediates alpha-MSH induced release of nucleus accumbens dopamine. Neuroreport 12: 2155-2158.

Lindemann B (2001) Receptors and transduction in taste. Nature 413: 219-225.

Liss B, Bruns R, Roeper J (1999a) Alternative sulfonylurea receptor expression defines metabolic sensitivity of K-ATP channels in dopaminergic midbrain neurons. EMBO J 18: 833-846.

Liss B, Franz O, Sewing S, Bruns R, Neuhoff H, Roeper J (2001) Tuning pacemaker frequency of individual dopaminergic neurons by Kv4.3L and KChip3.1 transcription. EMBO J 20: 5715-5724.

Liss B, Neu A, Roeper J (1999b) The weaver mouse gain-of-function phenotype of dopaminergic midbrain neurons is determined by coactivation of wvGirk2 and K-ATP channels. J Neurosci 19: 8839-8848.

Liu XH, Morris R, Spiller D, White M, Williams G (2001) Orexin a preferentially excites glucose-sensitive neurons in the lateral hypothalamus of the rat in vitro. Diabetes 50: 2431-2437.

Ludwig DS, Tritos NA, Mastaitis JW, Kulkarni R, Kokkotou E, Elmquist J, Lowell B, Flier JS, Maratos-Flier E (2001) Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance. J Clin Invest 107: 379-386.

Lund JP, Kolta A, Westberg KG, Scott G (1998) Brainstem mechanisms underlying feeding behaviors. Curr Opin Neurobiol 8: 718-724.

Maldonado-Irizarry CS, Swanson CJ, Kelley AE (1995) Glutamate receptors in the nucleus accumbens shell control feeding behavior via the lateral hypothalamus. J Neurosci 15: 6779-6788.

Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK (2001) Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol 435: 6-25.

Masukawa Y, Suzuki T, Misawa M (1993) Differential modification of the rewarding effects of methamphetamine and cocaine by opioids and antihistamines. Psychopharmacology Berl 111: 139-143.

Maurin Y, Banrezes B, Menetrey A, Mailly P, Deniau JM (1999) Three-dimensional distribution of nigrostriatal neurons in the rat: relation to the topography of striatonigral projections. Neuroscience 91: 891-909.

Merchenthaler I, Vigh S, Petrusz P, Schally AV (1982) Immunocytochemical localization of corticotropin-releasing factor (CRF) in the rat brain. Am J Anat 165: 385-396.

Meredith GE, Pennartz CM, Groenewegen HJ (1993) The cellular framework for chemical signalling in the nucleus accumbens. Prog Brain Res 99: 3-24.

Midgley LP, Wagstaff JD, Singh NA, Bush LG, Gibb JW, Hanson GR (1994) Dynamic dopaminergic regulation of neuropeptide Y systems in discrete striatal and accumbens regions. Eur J Pharmacol 251: 191-199.

Mignot E (1998) Genetic and familial aspects of narcolepsy. Neurology 50: S16-S22.

Mignot E, Nishino S, Guilleminault C, Dement WC (1994) Modafinil binds to the dopamine uptake carrier site with low affinity. Sleep 17: 436-437.

Miller JD, Farber J, Gatz P, Roffwarg H, German DC (1983) Activity of mesencephalic dopamine and non-dopamine neurons across stages of sleep and walking in the rat. Brain Res 273: 133-141.

Mirenowicz J, Schultz W (1996) Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. Nature 379: 449-451.

Mogenson GJ, Jones DL, Yim CY (1980) From motivation to action: functional interface between the limbic system and the motor system. Prog Neurobiol 14: 69-97.

Moriguchi T, Sakurai T, Nambu T, Yanagisawa M, Goto K (1999) Neurons containing orexin in the lateral hypothalamic area of the adult rat brain are activated by insulin-induced acute hypoglycemia. Neurosci Lett 264: 101-104.

Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD (1994) Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 8: 1298-1308.

Moyer JR, Brown TH (2002) Patch-clamp techniques applied to brain slices. In: Patch-clamp analysis: advanced techniques (Walz W, Boulton AA, Baker GB, eds), pp 135-194. Humana Press.

Nagai T, McGeer PL, McGeer EG (1983) Distribution of GABA-T-intensive neurons in the rat forebrain and midbrain. J Comp Neurol 218: 220-238.

Nakamura T, Uramura K, Nambu T, Yada T, Goto K, Yanagisawa M, Sakurai T (2000) Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. Brain Res 873: 181-187.

Nauta WJ, Smith GP, Faull RL, Domesick VB (1978) Efferent connections and nigral afferents of the nucleus accumbens septi in the rat. Neuroscience 3: 385-401.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS (2001) Mammalian sweet taste receptors. Cell 106: 381-390.

Neuhoff H, Neu A, Liss B, Roeper J (2002) I(h) channels contribute to the different functional properties of identified dopaminergic subpopulations in the midbrain. J Neurosci 22: 1290-1302.

Niijima K, Yoshida M (1982) Electrophysiological evidence for branching nigral projections to pontine reticular formation, superior colliculus and thalamus. Brain Res 239: 279-282.

Nishimura Y, Takada M, Mizuno N (1997) Topographic distribution and collateral projections of the two major populations of nigrothalamic neurons. A retrograde labeling study in the rat. Neurosci Res 28: 1-9.

Nishino S, Mignot E (1997) Pharmacological aspects of human and canine narcolepsy. Prog Neurobiol 52: 27-78.

Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E (2000) Hypocretin (orexin) deficiency in human narcolepsy. Lancet 355: 39-40.

Oades RD, Halliday GM (1987) Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. Brain Res 434: 117-165.

Okura M, Riehl J, Mignot E, Nishino S (2000) Sulpiride, a D2/D3 blocker, reduces cataplexy but not REM sleep in canine narcolepsy. Neuropsychopharmacology 23: 528-538.

Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS (1997) Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 278: 135-138.

Ookuma K, Sakata T, Fukagawa K, Yoshimatsu H, Kurokawa M, Machidori H, Fujimoto K (1993) Neuronal histamine in the hypothalamus suppresses food intake in rats. Brain Res 628: 235-242.

Overton PG, Clark D (1997) Burst firing in midbrain dopaminergic neurons. Brain Res Brain Res Rev 25: 312-334.

Packard MG, White NM (1990) Lesions of the caudate nucleus selectively impair "reference memory" acquisition in the radial maze. Behav Neural Biol 53: 39-50.

Paladini CA, Celada P, Tepper JM (1999) Striatal, pallidal, and pars reticulata evoked inhibition of nigrostriatal dopaminergic neurons is mediated by GABA(A) receptors in vivo. Neuroscience 89: 799-812.

Panula P, Yang HY, Costa E (1984) Histamine-containing neurons in the rat hypothalamus. Proc Natl Acad Sci U S A 81: 2572-2576.

Pessia M, Jiang ZG, North RA, Johnson SW (1994) Actions of 5-hydroxytryptamine on ventral tegmental area neurons of the rat in vitro. Brain Res 654: 324-330.

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18: 9996-10015.

Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, Maratos-Flier E (1996) A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature 380: 243-247.

Reid MS, Tafti M, Nishino S, Sampathkumaran R, Siegel JM, Mignot E (1996) Local administration of dopaminergic drugs into the ventral tegmental area modulates cataplexy in the narcoleptic canine. Brain Res 733: 83-100.

Richard D, Lin Q, Timofeeva E (2002) The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. Eur J Pharmacol 440: 189-197.

Richards CD, Shiroyama T, Kitai ST (1997) Electrophysiological and immunocytochemical characterization of GABA and dopamine neurons in the substantia nigra of the rat. Neuroscience 80: 545-557.

Robbins TW, Everitt BJ (1996) Neurobehavioural mechanisms of reward and motivation. Curr Opin Neurobiol 6: 228-236.

Romo R, Schultz W (1990) Dopamine neurons of the monkey midbrain: contingencies of responses to active touch during self-initiated arm movements. J Neurophysiol 63: 592-606.

Ronken E, Mulder AH, Schoffelmeer AN (1993) Interacting presynaptic kappa-opioid and GABAA receptors modulate dopamine release from rat striatal synaptosomes. J Neurochem 61: 1634-1639.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92: 573-585.

Saper CB, Chou TC, Elmquist JK (2002) The need to feed: homeostatic and hedonic control of eating. Neuron 36: 199-211.

Saper CB, Chou TC, Scammell TE (2001) The sleep switch: hypothalamic control of sleep and wakefulness. Trends Neurosci 24: 726-731.

Schultz W (1998) Predictive reward signal of dopamine neurons. J Neurophysiol 80: 1-27.

Schultz W (2002) Getting formal with dopamine and reward. Neuron 36: 241-263.

Schultz W, Apicella P, Ljungberg T (1993) Responses of monkey dopamine neurons to reward and conditioned stimuli during successive steps of learning a delayed response task. J Neurosci 13: 900-913.

Seiden LS, Sabol KE, Ricaurte GA (1993) Amphetamine: effects on catecholamine systems and behavior. Annu Rev Pharmacol Toxicol 33: 639-677.

Sergeeva OA, Korotkova TM, Scherer A, Brown RE and Haas HL (2003) Co-expression of non-selective cation channels of the transient receptor potential canonical family in central aminergic neurones. J Neurochem 85 (6): 1547-1552.

Sesack SR, Pickel VM (1992) Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. J Comp Neurol 320: 145-160.

Seutin V, Johnson SW, North RA (1993) Apamin increases NMDA-induced burst-firing of rat mesencephalic dopamine neurons. Brain Res 630: 341-344.

Seutin V, Massotte L, Renette MF, Dresse A (2001) Evidence for a modulatory role of Ih on the firing of a subgroup of midbrain dopamine neurons. Neuroreport 12: 255-258.

Seutin V, Mkahli F, Massotte L, Dresse A (2000) Calcium release from internal stores is required for the generation of spontaneous hyperpolarizations in dopaminergic neurons of neonatal rats. J Neurophysiol 83: 192-197.

Shalev U, Yap J, Shaham Y (2001) Leptin attenuates acute food deprivation-induced relapse to heroin seeking. J Neurosci 21: RC129.

Shepard PD, Bunney BS (1991) Repetitive firing properties of putative dopamine-containing neurons in vitro: regulation by an apamin-sensitive Ca(2+)-activated K+ conductance. Exp Brain Res 86: 141-150.

Shepard PD, German DC (1988) Electrophysiological and pharmacological evidence for the existence of distinct subpopulations of nigrostriatal dopaminergic neuron in the rat. Neuroscience 27: 537-546.

Sheriff S, Dautzenberg FM, Mulchahey JJ, Pisarska M, Hauger RL, Chance WT, Balasubramaniam A, Kasckow JW (2001) Interaction of neuropeptide Y and corticotropin-releasing factor signaling pathways in AR-5 amygdalar cells. Peptides 22: 2083-2089.

Simerly RB (1995) Anatomical substrates of hypothalamic integration. In: Rat Nervous System (Paxinos G, ed), pp 353-376.

Smith AD, Bolam JP (1990) The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. Trends Neurosci 13: 259-265.

Smith Y, Kieval J, Couceyro PR, Kuhar MJ (1999) CART peptide-immunoreactive neurones in the nucleus accumbens in monkeys: ultrastructural analysis, colocalization studies, and synaptic interactions with dopaminergic afferents. J Comp Neurol 407: 491-511.

Staley KJ, Otis TS, Mody I (1992) Membrane properties of dentate gyrus granule cells: comparison of sharp microelectrode and whole-cell recordings. J Neurophysiol 67: 1346-1358.

Stanford IM, Lacey MG (1996) Differential actions of serotonin, mediated by 5-HT1B and 5-HT2C receptors, on GABA-mediated synaptic input to rat substantia nigra pars reticulata neurons in vitro. J Neurosci 16: 7566-7573.

Stanley BG, Magdalin W, Seirafi A, Thomas WJ, Leibowitz SF (1993) The perifornical area: the major focus of (a) patchily distributed hypothalamic neuropeptide Y-sensitive feeding system(s). Brain Res 604: 304-317.

Steffensen SC, Lee RS, Stobbs SH, Henriksen SJ (2001) Responses of ventral tegmental area GABA neurons to brain stimulation reward. Brain Res 906: 190-197.

Steffensen SC, Svingos AL, Pickel VM, Henriksen SJ (1998) Electrophysiological characterization of GABAergic neurons in the ventral tegmental area. J Neurosci 18: 8003-8015.

Steketee JD, Kalivas PW (1991) Sensitization to psychostimulants and stress after injection of pertussis toxin into the A10 dopamine region. J Pharmacol Exp Ther 259: 916-924.

Stone EA, Cotecchia S, Lin Y, Quartermain D (2002) Role of brain alpha 1B-adrenoceptors in modafinil-induced behavioral activity. Synapse 46: 269-270.

Stratford TR, Kelley AE (1997) GABA in the nucleus accumbens shell participates in the central regulation of feeding behavior. J Neurosci 17: 4434-4440.

Stratford TR, Kelley AE (1999) Evidence of a functional relationship between the nucleus accumbens shell and lateral hypothalamus subserving the control of feeding behavior. J Neurosci 19: 11040-11048.

Stratford TR, Kelley AE, Simansky KJ (1999) Blockade of GABAA receptors in the medial ventral pallidum elicits feeding in satiated rats. Brain Res 825: 199-203.

Stratford TR, Swanson CJ, Kelley A (1998) Specific changes in food intake elicited by blockade or activation of glutamate receptors in the nucleus accumbens shell. Behav Brain Res 93: 43-50.

Suzuki T, Takamori K, Misawa M, Onodera K (1995) Effects of the histaminergic system on the morphine-induced conditioned place preference in mice. Brain Res 675: 195-202.

Svensson TH, Tung CS (1989) Local cooling of pre-frontal cortex induces pacemaker-like firing of dopamine neurons in rat ventral tegmental area in vivo. Acta Physiol Scand 136: 135-136.

Taber MT, Fibiger HC (1995) Electrical stimulation of the prefrontal cortex increases dopamine release in the nucleus accumbens of the rat: modulation by metabotropic glutamate receptors. J Neurosci 15: 3896-3904.

Taheri S, Sunter D, Dakin C, Moyes S, Seal L, Gardiner J, Rossi M, Ghatei M, Bloom S (2000) Diurnal variation in orexin A immunoreactivity and prepro-orexin mRNA in the rat central nervous system. Neurosci Lett 279: 109-112.

Tepper JM, Martin LP, Anderson DR (1995) GABAA receptor-mediated inhibition of rat substantia nigra dopaminergic neurons by pars reticulata projection neurons. J Neurosci 15: 3092-3103.

Thierry AM, Deniau JM, Herve D, Chevalier G (1980) Electrophysiological evidence for non-dopaminergic mesocortical and mesolimbic neurons in the rat. Brain Res 201: 210-214.

Tong ZY, Overton PG, Clark D (1996) Stimulation of the prefrontal cortex in the rat induces patterns of activity in midbrain dopaminergic neurons which resemble natural burst events. Synapse 22: 195-208.

Tschop M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. Nature 407: 908-913.

Turnbull AV, Rivier C (1997) Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. Proc Soc Exp Biol Med 215: 1-10.

van Bockstaele EJ, Pickel VM (1995) GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain. Brain Res 682: 215-221.

van den Pol AN, Gao XB, Obrietan K, Kilduff TS, Belousov AB (1998) Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. J Neurosci 18: 7962-7971.

Vorobjev VS, Sharonova IN, Haas HL, Sergeeva OA (2000) Differential modulation of AMPA receptors by cyclothiazide in two types of striatal neurons. Eur J Neurosci 12: 2871-2880.

Wagner U, Weiler HT, Huston JP (1993) Amplification of rewarding hypothalamic stimulation following a unilateral lesion in the region of the tuberomammillary nucleus. Neuroscience 52: 927-932.

Wang T, French ED (1995) NMDA, kainate, and AMPA depolarize nondopamine neurons in the rat ventral tegmentum. Brain Res Bull 36: 39-43.

Watanabe T, Taguchi Y, Shiosaka S, Tanaka J, Kubota H, Terano Y, Tohyama M, Wada H (1984) Distribution of the histaminergic neuron system in the central nervous system of rats: a fluorescent immunohistochemical analysis with histidine decarboxylase as a marker. Brain Res 295: 13-25.

Westwood SC, Hanson GR (1999) Effects of stimulants of abuse on extrapyramidal and limbic neuropeptide Y systems. J Pharmacol Exp Ther 288: 1160-1166.

Wightman RM, Zimmerman JB (1990) Control of dopamine extracellular concentration in rat striatum by impulse flow and uptake. Brain Res Brain Res Rev 15: 135-144.

Willesen MG, Kristensen P, Romer J (1999) Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology 70: 306-316.

Willie JT, Chemelli RM, Sinton CM, Yanagisawa M (2001) To eat or to sleep? orexin in the regulation of feeding and wakefulness. Annu Rev Neurosci 24: 429-458.

Wilson C, Nomikos GG, Collu M, Fibiger HC (1995) Dopaminergic correlates of motivated behavior: importance of drive. J Neurosci 15: 5169-5178.

Wilson CJ, Young SJ, Groves PM (1977) Statistical properties of neuronal spike trains in the substantia nigra: cell types and their interactions. Brain Res 136: 243-260.

Wise RA (1998) Drug-activation of brain reward pathways. Drug Alcohol Depend 51: 13-22.

Wise RA (2002) Brain reward circuitry: insights from unsensed incentives. Neuron 36: 229-240.

Wise RA, Rompre PP (1989) Brain dopamine and reward. Annu Rev Psychol 40: 191-225.

Wisor JP, Nishino S, Sora I, Uhl GH, Mignot E, Edgar DM (2001) Dopaminergic role in stimulant-induced wakefulness. J Neurosci 21: 1787-1794.

Wolfart J, Neuhoff H, Franz O, Roeper J (2001) Differential expression of the small-conductance, calcium-activated potassium channel SK3 is critical for pacemaker control in dopaminergic midbrain neurons. J Neurosci 21: 3443-3456.

Wolfart J, Roeper J (2002) Selective coupling of T-type calcium channels to SK potassium channels prevents intrinsic bursting in dopaminergic midbrain neurons. J Neurosci 22: 3404-3413.

Yamanaka A, Kunii K, Nambu T, Tsujino N, Sakai A, Matsuzaki I, Miwa Y, Goto K, Sakurai T (2000) Orexin-induced food intake involves neuropeptide Y pathway. Brain Res 859: 404-409.

Yamanaka A, Sakurai T, Katsumoto T, Yanagisawa M, Goto K (1999) Chronic intracerebroventricular administration of orexin-A to rats increases food intake in daytime, but has no effect on body weight. Brain Res 849: 248-252.

Yaswen L, Diehl N, Brennan MB, Hochgeschwender U (1999) Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat Med 5: 1066-1070.

Zahm DS, Grosu S, Williams EA, Qin S, Berod A (2001) Neurons of origin of the neurotensinergic plexus enmeshing the ventral tegmental area in rat: retrograde labeling and in situ hybridization combined. Neuroscience 104: 841-851.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. Nature 372: 425-432.