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Functional Diversity of Ventral Midbrain Dopamine and GABAergic Neurons

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Abstract

Recent findings indicate that VTA and SN dopaminergic (DA) and GABAergic neurons form subpopulations that are divergent in their electrophysiological features, vulnerability to neurodegeneration, and regulation by neuropeptides. This diversity can be correlated with the anatomical organization of the VTA and SN and their inputs and outputs. In this review we describe the heterogeneity in ion channels and firing patterns, especially burst firing, in subpopulations of dopamine neurons. We go on to describe variations in vulnerability to neurotoxic damage in models of Parkinson's disease in subgroups of DA neurons and its possible relationship to developmental gene regulation, the expression of different ion channels, and the expression of different protein markers, such as the neuroprotective marker calbindin. The electrophysiological properties of subgroups of GABAergic midbrain neurons, patterns of expression of protein markers and receptors, possible involvement of GABAergic neurons in a number of processes that are usually attributed exclusively to dopaminergic neurons, and the characteristics of a subgroup of neurons that contains both dopamine and GABA are also discussed.

Index Entries: substantia nigra; ventral tegmental area; orexin; hypocretin; Parkinson's disease; neuroprotection; development; TRP channels; calbindin.

Introduction

The heterogeneity of neuronal subpopulations within particular brain regions plays an important role in the information processing of those regions. Although it is of critical importance, the issue of heterogeneity has been largely ignored for aminergic nuclei; these neurons have been long considered as rather functionally homogenous populations. This is in spite of the fact that neurons within these nuclei often exhibit different connectivity profiles. In this review we will describe electro-

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physiological and molecular diversity within two midbrain dopaminergic nuclei—the substantia nigra (SN) and the ventral tegmental area (VTA). The neuromodulatory input from these nuclei on forebrain structures is known to shape many aspects of adaptive behavior, from working memory to motor control.

Recent findings indicate that VTA and SN dopaminergic (DA) and GABAergic neurons form subpopulations, divergent in their electrophysiological features, vulnerability (for DA cells) to neurodegeneration, and regulation by neuropeptides. Furthermore, this diversity is at least partly correlated with the anatomical organization of the VTA and SN and their inputs and outputs. In the beginning of this review we describe the divergent regulation of firing patterns, especially so-called burst firing, which is considered to be important for learning due to its appearance when novel environmental stimuli are presented. Possible correlations with the expression of different protein markers are reported. Next, we consider variations in vulnerability to neurotoxic damage in models of Parkinson's disease (PD) in subgroups of DA neurons and its possible relationship to developmental gene regulation and the expression of different ion channels. Neuroprotective markers such as calbindin (CB) and their pattern of expression are described. Whenever possible, the differences between neurons of SN and VTA will be shown, although many studies were made in one of these nuclei only, so comparison is not feasible. Similarly we will attempt to correlate, as far as possible, the regulation of DAergic and GABAergic activity. The contribution of the latter in regulation of a number of processes, such as reinforcement, that were previously attributed exclusively to DAergic transmission has been underestimated for a long time.

Dopaminergic SN pars compacta (SNC) neurons project mainly to the dorsal striatum, forming the mesostriatal system, and DAergic VTA neurons project mainly to the ventral striatum (nucleus accumbens, NAcc) and pallidum, prefrontal cortex, amygdala, and hippocampus, forming the mesocorticolimbic DA system (Fig. 1). The SNC is composed of two layers, the dorsal and ventral tiers, identified on the basis of dendritic arborization and connectivity patterns as well as CB-immunoreactivity. Dendrites of the SNC ventral tier DAergic neurons arborize in the SN pars reticulata (SNR) while the dendrites of dorsal tier cells have a predominantly mediolateral orientation. Nigrostriatal projections follow an

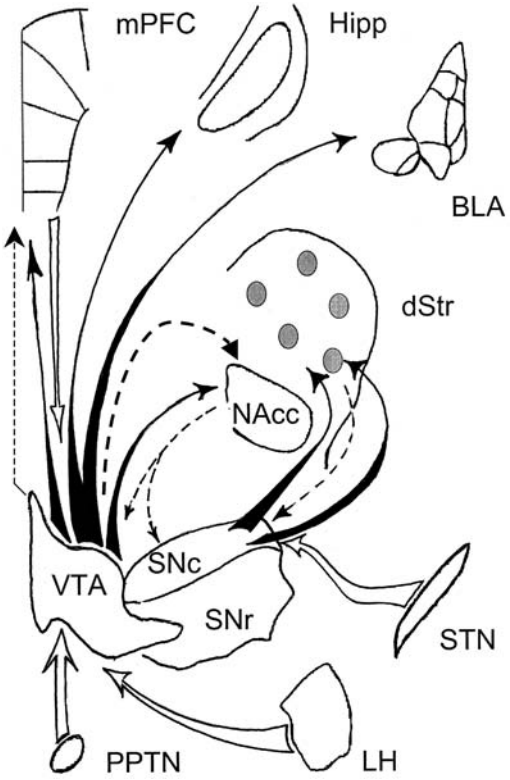

Fig. 1. A simplified scheme of the connectivity profile of VTA and SNc. Filled arrows indicate dopaminergic projections; dashed arrows, GABAergic; white arrows, glutamatergic pathways. Note that ventral portion of SNc projects to striatal patches (gray circles), while dorsal SNc, to striatal matrix. mPFC, medial prefrontal cortex; hipp, temporal hippocampus; BLA, basolateral complex of the amygdala; Nacc, nucleus accumbens; dStr, dorsal striatum; STN, subthalamic nucleus; PPTN, pedunculopontine tegmental nucleus, LH, lateral hypothalamic area.

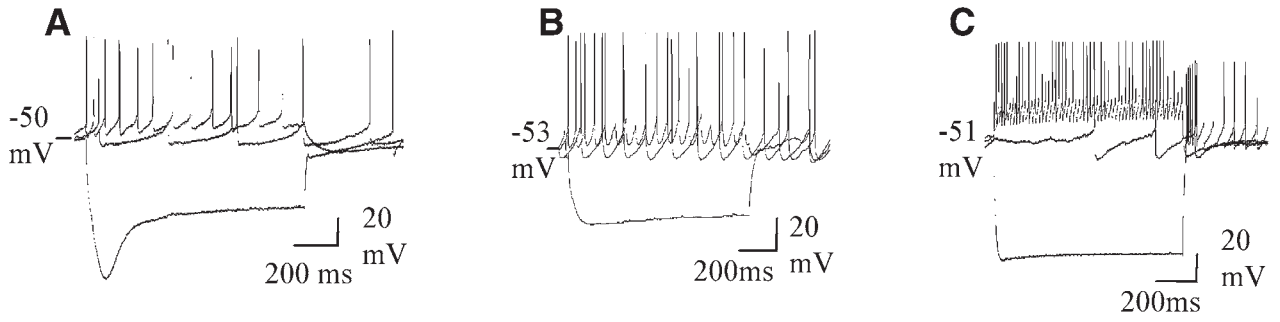

Fig. 2. Electrophysiological properties of the VTA neurons. **(A)** A dopaminergic VTA neuron: voltage responses to current pulses (–0.4 nA, 0 nA, +0.1 nA). **(B)** Example of a presumed GABAergic neuron with high spontaneous firing rate: voltage responses to current pulses (–0.3 nA, 0 nA, +0.1 nA). **(C)** Presumed GABAergic neuron with low spontaneous firing rate (from ref. *29,* Copyright 2003 by the Society for Neuroscience).

inverted dorsoventral topography, while in the VTA more medial aspects project to the shell of NAcc and more laterally to the NAcc core region. Further anatomical details are described by Fallon and Loughlin *(1).*

Regulation of DA Neuron Firing Modes

Electrophysiological properties of DAergic (sometimes termed "principal") cells in SN and the VTA have been studied extensively *(2–4).* Most of them are spontaneously active in vitro, their firing frequency is quite regular, and their action potential duration is longer than in GABAergic neurons. DA cells show pronounced time-dependent inward rectification, which is mediated by the activation of cyclic nucleotide-regulated cation (I_h) channels (Fig. 2A). In vivo, a second pattern of activity, burst firing—a repetitive occurrence of groups of action potentials with short interspike intervals—is frequently observed *(5).* Such firing results in a more efficient release of dopamine from both terminals and soma/dendrites than does regular firing (owing to the rapid saturation of the dopamine reuptake transporter) *(6).* The burst firing in both VTA and SN is associated with the presentation of novel rewarding stimuli or, as during conditioning, with reward predictors *(7,8).* About 75% of neurons have such responses in awake animals. Recent studies suggest a wider range of novel, adaptively relevant stimuli that elicit burst firing of DA neurons (*see* ref. *9* for review). Differences in the firing patterns of DA neurons in SN and VTA were found: Although the mean firing rate in these two nuclei was similar in chloral-hydrate-anesthetized rats, VTA neurons fired less regularly; 23% of the VTA neurons and only 3% of those in SN exhibited burst firing *(10).* On the other hand, about 75% of midbrain DA cells in awake nonhuman primates *(8)* and about 43% in rats *(11)* fire bursts.

A large body of evidence supports the necessity of excitatory afferent input for the induction of burst firing *(12).* Major excitatory inputs arise from the prefrontal cortex (PFC), the pedunculopontine tegmental nucleus (PPTN), the lateral hypothalamus (LH), and the subthalamic nucleus (STN). In vitro, burst firing can be induced by application of NMDA *(13).* The question if such in vitro burst firing is similar to natural burst firing is extensively discussed by Overton and Clark *(12).* NMDA-induced burst firing can be facilitated by the bee venom apamin: it increases the number of cells in which NMDA elicits burst firing (from 32% to 90%) and it makes the membrane potential oscillations that accompany burst firing appear more regularly *(14).* Apamin is a selec-

tive blocker of small-conductance, calcium-activated potassium channels (SK), which contribute to the medium afterhyperpolarization (AHP). The SK family consists of SK1–SK4 genes and SK3 is highly expressed in the midbrain *(15).* The density of apamin-binding sites is different in the SN and VTA *(16);* in the ventral tier of SN compacta the SK3 immunosignal was higher than in the dorsal tier or VTA. Selective blockade of these channels by apamin has a variety of effects on different DA neurons: it can increase the firing frequency, switch neurons to the burst mode, or have no effect. SK inhibition increases firing in a frequency-dependent manner. Activation of SK channels with 1-EBIO, a compound that increases the open state probability of these channels, is also increased at higher discharge frequencies *(17).* The firing-frequency-dependent effect of SK channels in control of excitability is likely to be linked to the higher intracellular Ca^{2+} concentration associated with higher firing rates.

SK channels determine the precision of the pacemaker-like firing of DA neurons, which is higher in the SN than in the VTA *(17).* An inhibition of SK channels decreases this precision, increasing the variability of firing. A correlation was found between the I_{AHP} amplitude and the SK3 immunosignal *(16).* In the VTA, inhibition or activation of SK3 channels did not change the precision of pacemaker-like firing or the firing frequency of DA neurons. However, the firing of DA neurons in the VTA was not as precise as in the SN *(17).* In chloral-hydrate-anesthetized rats, the activity of VTA neurons was less regular than that of SN neurons and a larger percentage of neurons showed burst firing (3% in SN vs 23% in VTA) *(10).* Thus, the mechanisms of regulation of pacemaker activity differ in SN and VTA.

Two considerations arise from the described differences in the tonic firing regularity of VTA/dorsal SNC tier vs ventral SNC tier neurons. Since the DA neuron represents a bi-stable dynamic system, cells firing in an irregular tonic mode are more likely to switch to the bursting mode of activity. This is in agreement with the higher ratio of bursting units found in VTA *(10)* and with a more normal distribution of interspike intervals in nonbursting than in bursting units in SNC of anesthetized rats *(18).* Another issue is the synchronization of DA cells. Increased variability of firing rates makes the entrainment of DA cells in the VTA and dorsal tier of SNC more problematic: only neurons with close initial firing rates could be easily synchronized by external inputs or within-regional interactions (for instance, via co-innervation by GABAergic cells, somatodendritic DA release or gap junctions). More regular firing in the ventral tier of SNC might predispose DA neuronal ensembles of one laminar channel (according to the innervation by SNR *[19]*) to synchronization; *see also* ref. *20.* This would boost DA signaling or/and trigger its oscillations in a corresponding part of the striatum due to the coordinated dynamics of transmitter release by synchronized units. Thus SK-channel-dependent variability of tonic firing might contribute to the differential involvement of VTA and SNC subregions in recently demonstrated oscillations in the basal ganglia circuits *(21,22).* Multisite in vivo recordings of midbrain DA neurons are necessary to prove this hypothesis.

SK3 channels mediate inhibitory postsynaptic potentials (IPSPs) evoked by metabotropic glutamate receptors (mGluR1)—a function of SK3 channels that is believed to be unique to the VTA. Activation of mGluR1 mobilizes Ca^{2+} from caffeine/ryanodine-sensitive stores and increases an apamin-sensitive potassium conductance *(23).* A recent study by Paladini et al. *(24)* demonstrates that α_1-receptor-mediated suppression of mGluR1-dependent IPSPs could be responsible for burst firing of DA neurons evoked by amphetamine.

Burst firing in some DAergic SN neurons in vitro could also be elicited by inhibition of T-type calcium channels (transient low-voltage-activated calcium channels), which are the primary calcium sources for activation of SK channels in SN *(25).* The most effective method to induce burst firing is a combination of SK and T-type channel inhibition. T-type channels, together with SK channels, control the preci-

sion of the DA SN pacemaker *(25).* In vivo intracellular recordings revealed that intracellular injection of calcium increases burst firing in SN DA neurons, whereas injection of the calcium chelator EGTA prevents it *(26).*

A recent coupled oscillator model of DA neurons suggests that irregular and burst firing can arise from dynamic interactions between the phases of the action potential (and intracellular calcium concentration) oscillations in somatic and dendritic compartments. Modulation of dendritic and somatic Ca^{2+}-currents could affect synchronization between oscillators during interspike intervals resulting in interval variability *(27).* Differential modulation of somatic and dendritic domains might be achieved by a specific pattern of cortical or/and striatal innervation. Indeed, burst firing missing in genetically DA-deficient mice is restored by L-dopa administration, suggesting the importance of a network feedback mechanism *(28).*

Lateral Hypothalamic (LH) Peptides, Orexins/Hypocretins, Elicit Bursting in VTA But Not SNC DA Neurons

Recent findings indicate that burst firing in vitro could also be elicited by orexins (hypocretins) *(29),* two neuropeptides that are expressed exclusively in the LH. Orexin A and B are both derived from preproorexin, a single polypeptide precursor, and are ligands of two G protein-coupled receptors, OX_1R and OX_2R *(30,31).* Orexin A activates both receptors with similar affinity, whereas orexin B is much more selective for the OX_2R. The administration of orexin A in vivo enhances arousal and locomotor activity as well as feeding, which is quite unusual, as most arousal-inducing substances are anorectic *(32,33).* It appears that whereas both orexin receptors seem to be involved in the regulation of arousal, OX_1R is mainly involved in the regulation of feeding—the increase in food intake induced by orexins is blocked by a selective antagonist of OX_1 receptors *(34).* In contrast, OX_2R mainly mediates the involvement of orexins in the regulation of the sleep–wake cycle. The mutation of OX_2R *(35)* or loss of orexin neurons *(36)* causes the sleep disorder narcolepsy, which is characterized by emotionally triggered loss of muscle tone, sleep-associated hallucinations, and excessive daytime sleepiness. The excessive daytime sleepiness is currently treated by psychostimulants that enhance dopaminergic tone. Orexins excite most, if not all, noradrenergic *(37),* serotonergic *(38,39),* and histaminergic neurons *(40).* Their effect on the dopamine system is more selective: they excite the majority of VTA DA neurons *(29)* but do not affect the firing rate of SNC DA neurons *(41).* In half of cells responding to orexins, these peptides caused an effect similar to that in other aminergic nuclei—an increase of firing rate, accompanied by depolarization. This was a direct postsynaptic effect, maintained in the presence of tetrodotoxin. In another subgroup of DA VTA orexin-responsive neurons, a switch from the regular firing pattern to an oscillatory one was observed, with alternation of periods of higher-frequency firing with silent periods. In some cells such oscillations were accompanied by burst firing—during periods of depolarization, bursts of action potentials *(3–6)* were observed (Fig. 3). An analysis of electrophysiological properties of the cells with different responses to orexins revealed that cells with oscillatory responses to orexins had significantly smaller AHP amplitudes *(29).*

A remarkable overlap was found between the expression of calbindin-D28k (CB) and orexin receptors *(29).* CB is a calcium-binding protein implicated in the regulation of the intracellular Ca^{2+} concentration. It is widely expressed in the whole brain, including forebrain, hindbrain, telencephalon, and midbrain *(42).* CB is expressed in 50% of neurons that express orexin receptors and is never detected in neurons that lack orexin receptors *(29).* Thus, the type of response to orexins could be associated with the presence or absence of CB.

The finding of orexinergic regulation of the VTA DA neuron firing enhances our under-

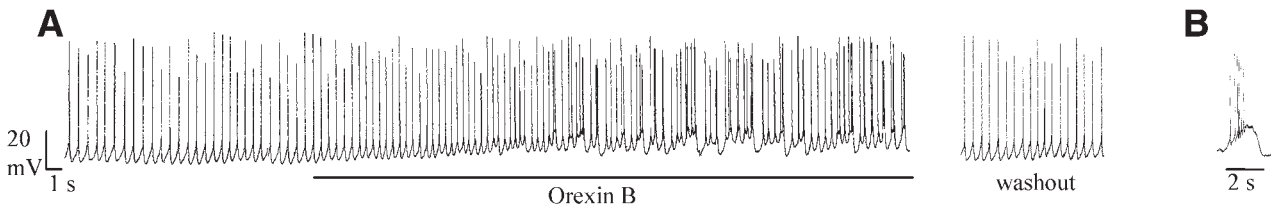

Fig. 3. **(A)** The application of orexin B (100 n*M*) caused burst firing in a subgroup of dopaminergic neurons in the VTA in vitro. **(B)** A typical orexin-mediated burst (from ref. *29,* Copyright 2003 by the Society for Neuroscience).

standing of how the lateral hypothalamus controls DA signaling. Indeed, orexins are produced only in the perifornical area of the LH. Orexin-immunoreactive fibers have been demonstrated in the VTA *(43)* and to a lesser extent in the SN *(41).* These fibers can utilize glutamate as cotransmitter *(44),* which has been shown to be released in a tetrodotoxin-sensitive manner in the VTA during lateral hypothalamic self-stimulation *(45).* Since the LH has long been considered as a substrate mediating the evaluation of primary reward *(46),* the orexinergic pathway might represent a means of excitation of a select population of VTA cells following presentation of relevant unconditioned stimuli. The findings that orexins can increase food intake and are sensitive to leptin, glucose, and ghrelin *(47)* suggests a preferential role for these neurons in food reward processing, although a more general involvement in the LH-VTA part of the reward circuitry cannot be ruled out.

Most VTA neurons are excited by both 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. A large body of evidence supports this hypothesis. Thus, the excessive daytime sleepiness of narcoleptics is currently treated with amphetamine-like compounds that enhance extracellular dopamine levels and the wake-promoting action of these compounds is absent in dopamine transporter knockout mice *(48).* Application of dopamine D_2 receptor agonists systemically or locally into the VTA exacerbate cataplexy, whereas D_2 receptor antagonists have the opposite effect *(49,50).* Cataplexy is elicited in narcoleptics by emotional arousal. In narcoleptic dogs (with a dysfunctional OX_2R), the most commonly used assay for cataplexy is the food-elicited cataplexy test *(51).* As the firing of VTA DA neurons is increased in response to primary rewards such as food *(7),* dysfunction of the orexin-regulation of dopamine neurons could be important for triggering of cataplexy.

The mechanism of orexin-induced excitation appears to be region-specific: In the locus coeruleus (LC), orexins decrease potassium conductance and the slow component of the AHP *(52);* in the tuberomamillary nucleus (TM) they act via the Na^+/Ca^{2+} exchanger *(40);* and in dorsal raphe (DR) a mixed cation channel is activated *(39).* The expression of orexin receptors correlates with expression of the TRP5 and TRP6 subunits of transient receptor potential canonical (TRPC) channels—a recently discovered family of Ca^{2+}-permeable cation channels *(53–55).* In GABAergic neurons of SN pars reticulata and VTA, orexin-evoked increases in firing rate are mediated via protein kinase A *(41),* but in DAergic neurons PKA inhibitors do not block excitations induced by orexins (Korotkova, unpublished observations). In preliminary experiments we found that orexins reduce the AHP in these neurons; however, the mechanism underlying the depolarization remains to be determined.

As described above, the expression of orexin receptors in DA neurons was correlated with the expression of the calcium-binding protein calbindin. Recently it has been shown that the

presence of CB in DA neurons correlates well with their electrophysiological properties and position either in SNC and VTA *(56).* In SNC only 17% of the neurons are CB-positive (CB+); these neurons display smaller I_h sag amplitudes and prolonged posthyperpolarization rebound delays. In the VTA, the percentage of CB+ and CB-negative (CB–) neurons is similar (~50%). VTA CB+ neurons have the smallest I_h sag, the longest rebound delay, the highest firing frequency, and the lowest AHP amplitude *(56).* The CB+ neurons in SNC and VTA possess smaller I_h currents. I_h channel inhibition prolongs the rebound delay in SNC CB– neurons but not in VTA CB+ cells. Therefore, a selective pacemaker control by I_h of the CB– subgroup has been suggested *(56).* SNC CB– DA neurons show an I_h channel-dependent transient postinhibitory excitation, VTA CB+ neurons a pronounced postinhibitory inhibition. DA neurons in vivo also display GABA-mediated indirect rebound excitation or direct inhibition *(57,58).* Cyclic nucleotide-regulated cation channels (HCN) that mediate I_h-current may differ in density in subsets of DA neurons in the VTA. The blockade of I_h-current with a relatively low (I_h-specific) dose of ZD7288 in the VTA in vitro leads to an inhibition of firing only in a subset of DA neurons *(59).* Thus, DA neurons exhibit differences in their firing patterns due to differential expression of SK, I_h, and T-type channels. In the following section we discuss how variations in the ionic dynamics may correlate with selective vulnerability of midbrain DA neurons.

Electrophysiological and Anatomical Features Predict Vulnerability of DA Neurons in Parkinson's Disease

It is well established that the neurodegeneration in Parkinson's disease (PD) and its animal models affects selected populations of DA cells in SNC and VTA. These differences in vulnerability partially correlate with or may even be explained by the specific regulation of their excitability and by the anatomical localization of these neurons.

Determinants of differential vulnerability of midbrain DA neurons have been elucidated by studies of neurodegeneration induced by the long-known neurotoxins MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-OHDA (6-hydroxydopamine), as well as by the more recently discovered rotenone, an agricultural toxin that produces Parkinsonism in animals and humans. All these toxins, especially rotenone, elicit an inhibition of the mitochondrial complex 1, which is critical in the control of oxidative phosphorylation *(60,61).* DA neurons are selectively sensitive to mitochondrial complex 1 inhibition *(62);* the reason for such selectivity is unknown. Reduced activity of the mitochondrial complex 1 is found in PD patients as well *(63).* Modeling this effect by rotenone-induced partial inhibition of the complex 1 causes activation of ATP-sensitive potassium (K_{ATP}) channels in 40% of DAergic SNC neurons *(64).* The remaining DA SNC neurons require a 100-fold higher dose of rotenone for a comparable activation of K_{ATP} channels, demonstrating a lower sensitivity of these cell populations to mitochondrial complex 1 inhibition. K_{ATP} channels are closed at high ATP and low ADP levels and open in the presence of a decreased ATP/ ADP ratio. Their activation causes hyperpolarization, thereby reducing neuronal firing and calcium influx. They are octameric proteins consisting of four pore-forming Kir 6 (members of an inwardly rectifying potassium channel family) and four regulatory SUR (sulfonylurea receptor, members of the ATP-binding cassette transporter superfamily) subunits. K_{ATP} channels are widely expressed throughout the CNS, especially in SN *(65).* The nonuniform responsiveness of K_{ATP} channels in DA neurons to rotenone-induced metabolic stress correlates with the expression of SUR subunits of K_{ATP} channels. Less-responsive cells possess the SUR 2B isoform in combination with Kir 6.2, whereas more-responsive neurons express the SUR 1 isoform and Kir 6.2.

In weaver mice, which are mutants in G protein-gated inwardly rectifying potassium channels (GIRK2) and show a PD-like pattern of DA cell loss *(66),* the Sur2B subunit in combination with Kir 6.2 (the phenotype with a

lower responsiveness to metabolic stress) is not expressed. Interestingly, SNR GABAergic neurons, which do not degenerate in PD, express exclusively the SUR1 subunit *(64).* This indicates a higher resistance to metabolic stress in the population of DA cells in the SNC that are readily hyperpolarized in response to an altered ATP to ADP ratio. It is not clear, however, whether the same rule describes vulnerability of VTA DA neurons. Notably, all SN DA neurons in weaver mice (Sur1 and Kir 6.2 composition of K_{ATP} channels) are CB+, but in wild-type mice CB+ neurons can express both types of SUR subunits *(67).*

Cell loss in the MPTP and 6-OHDA models of PD is confined to CB-positive midbrain DA neurons *(68,69).* The neurotrophin glial cell-line-derived neurotrophic factor (GDNF) significantly increased the number of surviving DA neurons and increased the density of CB-positive neurons but did not affect the density of calretinin-positive neurons in culture *(70).* However, in calbindin null-mutant mice, the loss of neurons after MPTP treatment was similar to the wild-type *(71).* Calbindin-deficient weaver mice also show a loss of neurons similar to weaver mice. Thus, resistance of CB+ neurons cannot be directly explained by expression of calbindin. It appears that is not required for protection, but rather serves as a marker of less vulnerable neurons *(71).* Neurons that lack calbindin are vulnerable to Ca^{2+}-related injury and, as we have described above, differ in their expression of ion channels from calbindin-containing neurons. Calbindin is also implicated in pathophysiology in other brain areas. After the induction of kindling-induced epilepsy, granule cells from the dentate gyrus show a significant loss of CB. Granule neurons from kindled rats exhibit a markedly enhanced Ca^{2+}-dependent inactivation of Ca^{2+} currents, and a smaller whole-cell Ca^{2+} current *(72).* The loss of CB from a neuron could reduce Ca^{2+} entry in order to make it less excitable *(73).*

Both the localization in the mesencephalon and the connectivity pattern of degenerating DA neurons differs from resistant ones. First of all, cells of the dorsal tier of SNC are less affected in models of PD. This might be explained simply by CB-specific neuronal loss, since the dorsal tier contains preferentially CB+ cells. The CB– cells of the ventral tier of SNC projecting to striatal patches (striosomes) are selectively sensitive to 6-OHDA *(74).* These neurons are first lost in PD and after MPTP-treatment; however, VTA neurons are also partially lost in PD and its MPTP *(75)* and 6-OHDA models *(69).* Interestingly, the dorsal tier of SNC and VTA cells projecting to the shell of NAcc are less affected in PD models *(76)* and receive relatively few striatal inhibitory projections when compared with the ventral tier of SNC *(1).* Furthermore, there is a distinct pattern of GABAergic dendritic innervation of the ventral tier DA neurons by SNR neurons *(1).* The predominant postinhibitory excitation in the CB– DA cells located in the ventral tier, due to higher expression of I_h channels, fits the more pronounced striatal feedback inhibition. On the other hand, higher postinhibitory excitability might lead to higher metabolic demands on CB– cells. A combination of these factors, together with reduced responsiveness of K_{ATP} channels, would render a neuron more vulnerable to metabolic stress. An initial loss of a population of the most vulnerable cells leads to increased subthalamic nucleus excitatory input on the rest of the SNC population caused by an evolving imbalance in basal ganglia circuitry *(77).* This results in the cascade-like degeneration of less vulnerable DA neurons and PD progression.

Genes That Regulate Development and Survival of Midbrain DAergic Neurons

The most recent models of mesencephalic DA cell-loss exploit knockout of genes responsible for the development of this neuronal population. Several transcription factors are known to regulate the differentiation of DAergic neurons. Sonic hedgehog and Fgf8 factors control their fate in the anterior neural plate

(78), expression of other early factors—e.g., Lmx 1b, En1/2 appears before the key DA synthesis enzyme, tyrosine hydroxylase; the orphan nuclear receptor Nurr1 and homeobox gene Pitx3 also anticipate expression of TH. Expression of Nurr 1 continues in mature dopaminergic neurons. Ablation of Nurr1 in mice leads to absence of TH and agenesis of midbrain DA neurons. Saucedo-Cardenas et al. *(79)* showed that ventral midbrain DA neurons require Nurr1 for their final differentiation. This defect is specific to midbrain dopamine although Nurr 1 is also expressed in other limbic structures. Expression of TH in the periglomerular region of the olfactory bulb is unaffected in Nurr1-null mutant mice, though these neurons express Nurr1 in wild-type mice. The onset of Pitx3 expression is not altered in Nurr1−/− mice but neonates display a significant loss of Pitx3-positive midbrain cells. Nurr1 could be a regulator of genes that promote dopamine survival, including CB, brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) *(79).*

Pitx3, but not Nurr1, activates the mouse TH promoter through a direct interaction with a high-affinity binding site *(80).* Expression of Pitx3 is more selective than Nurr1 and is limited to the midbrain and eye. Pitx3 is expressed in the ventral tier of SNC and in half of VTA neurons *(81).* The Pitx3-positive neuronal population is more susceptible to degeneration. The number of Pitx3 neurons is decreased in patients with PD and in 6-OHDA-lesioned rats. Unlike the situation with Nurr1, ablation of Pitx3 differentially affects DA neurons depending on their localization. The DA cell population in VTA and A8 of newborn aphakia (ak), Pitx3-knockout mice that have reduced spontaneous locomotor activity *(81)* is intact but SNC cells are absent *(82).* TH-immunoreactivity was nearly absent in 6-wk-old mice—the dorsal and ventral tiers of SNC and SNR were more markedly affected than SNC pars lateralis. Dopamine and DOPAC but not norepinephrine levels are also reduced *(82).* Van Den et al. *(81)* describe that whereas on embryonic day 12.5 (E12.5) there are no differences between ak and wild-type, in ak newborns (P1) the SN is devoid of TH-positive cells and VTA is not affected. However, at day P100 half of the ak VTA neurons are lost, though normally the number of the DA neurons in SN and VTA does not significantly change during postnatal development *(83).* Thus, expression of Pitx3 plays an important role in mature VTA neurons and is required for the development of SN neurons and for the survival of a subset of DAergic VTA neurons. Knockouts of Pitx3, ak mice have been proposed as a possible model of Parkinson's disease *(81).*

Pitx3 is homologous to two other homeobox proteins, Pitx1 and Pitx2—together they constitute a distinct subfamily of paired-like homeoproteins *(84).* Pitx1 is expressed outside the developing CNS *(85).* Pitx2 is expressed in developing diencephalon and mesencephalon and is associated with GABAergic neurogenesis *(86).* The Pitx2 gene and the Gad 1 gene, which codes for 67 kDa glutamate decarboxylase, the key enzyme of GABA synthesis, are expressed in similar patterns (E 11.5–E 12.5). The expression of Pitx2 is not transient; it remains in newborns as well *(86).* The Gad 1 promoter has a specific binding site for Pitx2 and the Pitx2-activated Gad1 promoter *(87).*

Another homeodomain transcription factor involved in the development of DA cells is the so-called Engrailed (En) protein. In rats there are two homologs, En-1 and En-2, which have different expression profiles in subgroups of DA neurons *(88).* En-1 is highly expressed by most DA neurons in SNC and VTA, En-2 displays high expression in a subset of them and low expression in the remainder. In En-double mutant mice midbrain DAergic neurons are generated during embryonic ontogenesis but disappear by E14. Observations of single (En-1 or En-2) mutants revealed that En1 and En2 compensate for the loss of each other. In normal rats DA neurons continue to express both En in adults. Expression of the α-synuclein gene, which carries a point mutation in familial PD *(89),* is controlled by En. α-Synuclein is absent in double En-mutants at E12, when

midbrain neurons are still present *(88)*. Thus knockouts of developmental genes can be used for modeling essential features of PD. However, it is not yet clear which other genes and factors are regulated by the described genes, whether they regulate and compensate for each other and whether their function is disturbed in patients with PD.

Midbrain GABAergic Neurons

The second class of neurons in the SN and VTA are GABAergic neurons. The functional importance of VTA GABA neurons has been underestimated for a long time and was considered to be mainly limited to inhibition of DAergic neurons. However, these neurons also contribute to mesocorticolimbic connections: 58% of mesoprefrontal neurons and 20% of mesoaccumbal neurons are GABAergic *(90)*. Moreover, a projectional selectivity of input from the PFC to the VTA was found: the PFC sends projection to DAergic mesocortical neurons and GABAergic mesoaccumbal neurons *(90)*. The stimulation of the NAcc as well as administration of the NMDA antagonists APV or MK-801 caused a decrease of firing rate in GABAergic VTA neurons in halothane-anesthetized rats *(91)*. The excitatory synapses on DA and GABA VTA neurons are different: synapses on DA cells exhibit a depression in response to repetitive activation, whereas synapses on GABA cells show a facilitation. Synapses on DA cells express NMDA-dependent LTP but excitatory synapses on GABAergic neurons do not. Synapses on DA cells are not affected by the $GABA_B$ receptor agonist baclofen, while synapses on GABA neurons are depressed by it *(92)*.

The place of GABAergic VTA neurons in reward circuitry is not clear. On the one hand, inhibition of GABA neurons leads to disinhibition of DA VTA neurons. Whereas some drugs of abuse (such as psychostimulants) increase dopamine release in the midbrain directly, others (e.g., opiates) do not directly affect dopamine neurons in vitro but hyperpolarize presumed GABAergic neurons reducing spontaneous GABA-mediated input to DA cells, i.e., disinhibition *(93)*. Cannabinoid receptor agonists inhibit GABAergic inhibitory postsynaptic currents (CB_1 cannabinoid receptor-mediated) but also do not affect the DA neurons directly. Thus, it has been hypothesized that inhibition of GABAergic neurons by cannabinoids could lead to increased firing of DAergic VTA neurons *(94)*.

On the other hand, it was recently found that several addictive compounds affect GABA and DA neurons in a similar rather than a reciprocal manner. Another drug of abuse, nicotine, increases the firing rate not only of DA neurons but also of GABA neurons in the VTA *(95)*. In GABAergic neurons a quick desensitization was observed—although the firing rate of GABA cells is increased after a single-dose application of nicotine (mimicking the increase in nicotine in a smoker's brain), it was not increased in response to continuous application of nicotine. The response in DAergic cells was stronger and desensitization was not as fast *(95)*. Activation of nicotinic acetylcholine receptors (nAChRs) on presynaptic terminals in the VTA enhances glutamatergic inputs to DA neurons and can induce LTP of the excitatory inputs (these effects are α-7 subunit-mediated) *(96)*. Interestingly, there is a difference in distribution of nicotinic receptor subunits both between DAergic and GABAergic neurons and in neurons in VTA and SN (class-specificity and region-specificity): the component of the response, which is not markedly desensitized, is mediated by α-7 nAChRs that are more highly expressed in the VTA (about 40% of DA and GABA neurons *[97]*) than in the SN. The slow-current component, which is mainly mediated by the more highly expressed β-2 nAChR (similar expression in SNC and VTA), becomes desensitized *(98)*. Nicotine-elicited currents in DA neurons in VTA have larger amplitudes than those in SNC *(97)*. Low doses of nicotine may have neuroprotective properties: the incidence of PD is lower in smokers than in nonsmokers *(99)*. Both acute and chronic administration of low doses of nicotine

in 6-OH-DOPA and MPTP-treated rats and mice lead to neuroprotection, but α_4 nAChR knockouts lack such neuroprotection. Only low but not high doses caused neuroprotection suggesting that nicotine-induced activation rather then desensitization is responsible for such effect *(100).* Detailed information concerning the nicotinic role in PD can be found in ref. *101.*

Differences in the molecular composition of nicotinic receptor subtypes in GABAergic neurons correlate with difference in their electrophysiology. Whereas in the VTA the major subclass of GABA neurons exhibits an accommodating firing pattern during depolarizing pulses, the majority of GABA neurons in the SN does not show accommodation *(97).* The waveforms of ACh-elicited currents correlates with differences in the response to depolarization: in nonaccommodating GABA neurons the waveform is similar to DA neurons—large amplitude, slow rounded peak, lacking a pronounced decay component—whereas in accommodating VTA GABA neurons the peak current is reached quickly and decays more slowly *(97).* The functional consequences of such differences are unknown.

We discriminated two subgroups of GABAergic neurons in the VTA according to their firing rate *(29).* One group fired at a relatively high frequency, similar to SN GABA neurons (~8 Hz); the cells in the second group fired slowly (~0.7 Hz). Both groups of cells displayed a similar spike duration (shorter than in DA neurons), regular firing (no bursts or silent episodes were observed), and fired at high frequency (>30Hz) during depolarizing current steps *(29)* (Fig. 2B,C). No differences were found in their ability to accommodate firing during depolarization, such as those described in ref. *97.* It is unlikely that the reason for slow firing was hyperpolarization (for example, because of inhibitory input), since slow-firing cells had more positive spike threshold (–52 and –49 mV respectively). Slow-firing cells had a larger AHP amplitude (14 vs 11 mV in fast-firing cells).

Both subgroups of GABAergic neurons were uniformly excited by orexins. The firing of slow cells was increased to a greater extent (relative to the initial firing rate) and the maximal firing frequency in the presence of orexins approached the rate seen in fast-firing cells. This effect, like the previously described orexin-induced excitation of DA VTA neurons, was postsynaptically mediated *(29).* Thus, orexins, like nicotine, excite both DA and GABA VTA neurons in vitro. The action of orexins on VTA neurons in vivo has not yet been studied, so the net effect of such simultaneous excitation of two neuron classes is not known. However, it was recently found *(102)* that GABAergic VTA neurons are also activated during brain stimulation reward: the activity of these cells is increased approx 2 s before active (nosepoke) intracranial self-stimulation of the medial forebrain bundle but not after passive stimulation. Immediately after stimulation the activity of these neurons was decreased relative to baseline levels. In contrast to recordings from DA neurons during intracranial self-stimulation, no adaptation of the response of GABAergic cells was found. Heroin self-administration produced a similar action on VTA non-DA neurons *(103).* Steffensen et al. hypothesize that GABA VTA neurons may play a role in attending to rewarding stimuli *(102).* At the present time it is difficult to say which group of GABAergic cells recorded in vitro is involved in such processing—it is not easy to distinguish these subgroups in vivo, because all GABAergic neurons recorded in halothane-anesthetized rats possess a high firing frequency (~19 Hz) and no accommodation after depolarizing pulses in vivo was observed *(91).* The pattern of their activity was characterized by alternation of a 0.5–2 s "on" period, accompanied by depolarization (~9.4 mV), and 0.5–2 s "off" periods. Furthermore, in freely moving rats the firing rate of VTA non-DA neurons, in contrast to that of DA neurons, depends on the phase of the sleep–wake cycle *(91).*

GABAergic neurons in SN pars reticulata inhibit the thalamocortical motor system and medial pontine reticular formation *(104–106).* Increased firing of GABAergic substantia nigra

pars reticulata neurons in patients with PD is associated with catalepsy—an enhanced muscle tone *(106).* The response to dopaminergic degeneration differs in various subgroups of these cells. In rats under chloral hydrate anesthesia several firing patterns in GABAergic neurons of SN pars reticulata and compacta were observed: irregular firing (in the majority of cells, 68%), regular firing (18%), and burst firing. The firing rates of regular firing cells were higher than in the latter two groups, suggesting an inhibitory input to neurons with irregular and burst firing. The firing rate of bursting cells was the lowest. After an ipsilateral 6-OHDA lesion of DA cells, the firing rate of irregular and bursting cells, as well as the proportion of bursting cells, increased, whereas the firing rate of regular cells was not affected. Contralateral to lesion, the firing rate of all three subgroups increased *(107).*

The picture of just two types of VTA neurons—DA neurons inhibited by dopamine and non-DA neurons, presumed GABAergic, inhibited by the opioid peptides DAMGO or Met-enkephalin *(93)*—is clearly too simple. First, 6-OHDA lesions decrease opiate receptor binding by 20% *(108);* second, a subset of neurons (~27%) inhibited by both dopamine and met-enkephalin was described. Their shape and spontaneous frequency is similar to that of "principal" (DA) cells *(109).* Single-cell RT-PCR studies revealed that a considerable proportion of DA (tyrosine hydroxylase-positive) cells express GAD (glutamic acid decarboxylase, a marker of GABAergic cells) as well. Klink et al. *(97)* found that up to 27% expressed both TH and GAD; such coexpression depends on age and is maximal at PD16–17. They did not find a dependence of such phenotype on the electrophysiological class or location of the cell. Similarly, we showed recently that 13% of VTA neurons were both TH-and GAD65-positive in 3–4-wk-old rats. Their size was equal to TH-positive, GAD-negative (DA) cells and significantly larger than that of TH-negative, GAD-positive (GABA) cells. All cells of this group expressed at least one subtype of orexin receptor; none of them expressed CB *(29).*

Using a combination of immunocytochemistry, *in situ* hybridization, and retrograde tracing, it was found that 10% of mesostriatal DA neurons lying in the medial region of SN compacta and VTA, contained GAD65 mRNA *(110).* Interestingly, only GAD65 that is present as an inactive apoenzyme, providing a reservoir of GAD but not GAD67, the active form, saturated with the cofactor, was colocalized with DA. Such colocalization may be important for neuroprotection during excessive activity *(110).*

Conclusions

To summarize, subpopulations of midbrain dopamine and GABAergic neurons differ in their spontaneous firing rate, firing pattern, and maximal firing frequency. These differences are caused by the differential expression of membrane ion channels regulating excitability. In dopamine neurons, where this issue has been examined in detail, differences in the expression of the channels responsible for I_{AHP}, I_h, and I_T currents have been shown to be involved in this diversity. The calcium-binding protein calbindin is a marker for neuronal subpopulations containing particular combinations or expression levels of these ion channels. Differential expression of these channels affects the regulation of firing of DA neurons by afferent inputs such as from the lateral hypothalamic orexin/hypocretin system. In addition to regulating firing, differential expression of channels mediating I_{AHP}, I_h, and I_T, together with the channels responsible for hyperpolarization in the presence of low ATP levels (K_{ATP} channels), are likely to contribute to selective vulnerability of DA neuron subpopulations in Parkinson's disease. Specification of different subpopulations and their specific ion channels is likely to be determined during development and controlled by genes such as Pitx3 and Engrailed.

Subpopulations of GABAergic neurons are also present in the VTA and SN. They differ in their firing frequency, expression of nicotinic

acetylcholine receptors, and projection patterns. Some of these neurons appear to play a role in attention/arousal and reward processes. Finally, evidence is accumulating for a third class of neurons in the VTA/SN, which coexpress the synthesizing enzymes for dopamine and GABA. Modulation of forebrain networks is carried out by heterogeneous neuronal classes in the ventral midbrain, resulting in coherent DA and GABA impact on target neuronal populations and an adaptive shaping of their activity.

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