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2015•2016
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Activation of glycine receptors decreases pacemaking activity in midbrain dopamine neurons independent of the alpha 2 subunit

Promotor :
Prof. dr. Bert BRONE

Copromotor :
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Jens Devoght
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Acknowledgements

My senior internship is now at its end, but not even close my interest in electrophysiology.

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Thank you,

Jens Devoght

List of Abbreviations

aCSF	artificial cerebrospinal fluid
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Ct	threshold cycle
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid receptor type A
$GABA_B$	γ-aminobutyric acid receptor type B
$GABA_C$	γ-aminobutyric acid receptor type C
GIRK	G protein-coupled inwardly-rectifying potassium channels
Glra	mouse gene glycine receptor alpha subunit
Glrb	mouse gene glycine receptor beta subunit
GlyR	glycine receptor
GlyRα	glycine receptor alpha subunit
GlyRα2KO	glycine receptor alpha 2 knock-out
GlyRβ	glycine receptor beta subunit
HCN	hyperpolarization-activated cyclic nucleotide-gated
Hprt1	mouse gene hypoxanthine phosphoribosyltransferase type 1
I_h	inward hyperpolarization-activated cation current
NDS	normal donkey serum
NMDA	N-methyl-D-aspartate
PBS	phosphate-buffered saline
PFA	paraformaldehyde
Pgk1	mouse gene phosphoglycerate kinase type 1
RNA	ribonucleic acid
SK	small-conductance, calcium-activated potassium channels
SNc	substantia nigra pars compacta
TM	transmembrane domain
VTA	ventral tegmental areas
WT	wild-type

Abstract

Dopamine is one of the main neurotransmitters in the brain and a tight modulation of its release is essential for a proper brain function. Dopamine is released into the forebrain and contributes to reward-motivated behavior, cognition and motor control. Therefore, dysfunction of dopamine signaling is associated with various diseases, such as Parkinson's disease, psychosis and drug abuse. The dopamine-releasing neurons reside in the midbrain, where their activity is modulated by different neurotransmitters. A better understanding of these modulatory mechanisms on dopamine release is essential for unveiling the etiology of these diseases and development of new treatment strategies.

The neurotransmitter glycine plays a major role in the modulation of dopamine neuron activity, yet it is unclear which subunits are involved. To address this gap, we first confirmed the effect of glycine on basal dopamine neuron firing in the substantia nigra pars compacta (SNc) within the midbrain. Next, since preliminary data from our lab indicate a significant role for the alpha 2 subunit of the glycine receptor (GlyRα2) in dopaminergic signaling, we repeated these experiments in GlyRα2 knock-out littermates.

We performed loose cell-attached voltage-clamp recordings on dopamine neurons in the SNc in brain slices from adult wildtype mice. We showed that application of 1 mM glycine significantly decreases autonomous firing, which indicates that glycine at synaptic concentrations has a strong inhibitory function. We next sought to determine putative distinct roles of the glycine receptor subunits. First, we established the presence of all subunits in the midbrain, using real-time PCR. Next, immunohistochemical co-staining revealed the presence of GlyRα2 on dopamine neurons, yet electrophysiological measurements showed no modulatory effects of GlyRα2 on baseline dopamine neuron activity or in response to glycine application at synaptic concentrations. However, it is conceivable that high-affinity GlyRα2s are involved in the modulation at lower, tonic glycine concentrations and/or in burst firing modulation.

Our findings clearly demonstrate the involvement of glycine receptors in modulation of dopamine neuron activity, but modulation was independent of GlyRα2s at baseline activity and activity in presence of synaptic glycine concentrations. This can contribute to better insights into the etiology of dopamine-related diseases, such as schizophrenia and drug abuse, and development of new treatment strategies. Elucidating the distinct roles of the different subunits on dopamine neuron activity in future research will contribute even more.

Samenvatting

Dopamine is één van de belangrijkste neurotransmitters in de hersenen. Een strikte modulering van de vrijzetting van dopamine is dan ook essentieel voor een correcte hersenfunctie. Deze vrijzetting in de voorhersenen draagt bij aan beloning-gemotiveerd gedrag, cognitie en motorische controle. Om deze reden kan een disfunctie van dopamine-signalering leiden tot verschillende aandoeningen, zoals de ziekte van Parkinson, psychose en drugsmisbruik. De dopamine neuronen zijn gelokaliseerd in de middenhersenen, waar hun activiteit gemoduleerd wordt door verschillende neurotransmitters. Een betere kennis van deze modulerende mechanismen op dopamine-vrijzetting is essentieel voor het ontsluieren van de ontstaansredenen van deze diverse ziekten en de ontwikkeling van nieuwe behandelingsstrategieën.

De neurotransmitter glycine speelt een belangrijke rol in de modulatie van dopamine neuron activiteit, maar het is echter onduidelijk welke subeenheden betrokken zijn. Om dit uit te klaren hebben we allereerst het effect van glycine op de basale activiteit van dopamine neuronen in de volwassen middenhersenen bepaald. Hierbij werd de focus gelegd op de hersenregio substantia nigra pars compacta (SNc). Vervolgens werden deze experimenten herhaald voor GlyRα2 knock-out nestgenoten. Dit omdat preliminaire onderzoek een significante rol aangaf voor de alfa 2 subeenheid van de glycine receptor (GlyRα2) in dopaminerge signalisatie.

We voerden *loose cell-attached voltage-clamp* metingen uit op dopamine neuronen van de SNc in hersencoupes van volwassen wild-type muizen. Hierbij werd aangetoond dat de toediening van 1 mM glycine de pacemaker activiteit van dopamine neuronen significant vermindert. Dit geeft aan dat glycine op synaptische concentraties een sterk remmende functie uitoefent. In een volgende stap werd onderzocht welke afzonderlijke rollen de verschillende glycine receptor subeenheden uitoefenen. Ten eerste hebben werd de aanwezigheid van alle subeenheden in de middenhersenen aangetoond door middel van real-time PCR. Vervolgens onthulde een immunohistochemische co-kleuring de aanwezigheid aan van GlyRα2 op dopamine neuronen, alhoewel elektrofysiologische metingen geen modulerende effecten aantoonden van GlyRα2 op de basale activiteit van dopamine neuronen of in respons op glycine applicatie in synaptische concentraties. Toch is het echter aannemelijk dat de hoge-affiniteit GlyRα2en wel betrokken zijn in de modulatie bij lagere tonische glycine concentraties en/of modulatie van de fasische activiteit.

Onze bevindingen tonen duidelijk aan dat glycine receptoren betrokken zijn in de modulatie van dopamine neuron activiteit, maar dat dit onafhankelijk gebeurt van GlyRα2en in basale activiteit en activiteit in aanwezigheid van synaptische concentraties aan glycine. Deze waarnemingen dragen dan ook bij tot een beter inzicht in de etiologie van dopamine-gerelateerde ziekten, zoals schizofrenie en drugs, en de ontwikkeling van nieuwe behandelingsstrategieën. Toekomstig onderzoek in functionaliteit van de verschillende subeenheden in dopamine neuronen zal een nog sterkere bijdrage kunnen leveren.

X

1. Introduction

1.1. Dopamine

Dopamine is a catecholamine acting as one of the main neurotransmitters in the brain (1). It is released by dopamine neurons residing in the ventral tegmental areas (VTA) and substantia nigra pars compacta (SNc) in the midbrain. These neurons project to different areas in the brain, such as the prefrontal cortex and striatum, and contribute to reward-motivated behavior, cognition and motor control (1). Dysfunction of the dopaminergic system is associated with various diseases, such as Parkinson's disease, psychosis and drug abuse (2). Hence, a tight modulation of dopamine release is essential for proper brain function, and unveiling the regulatory mechanisms is essential towards a better understanding of the diseases and development of new treatment strategies.

1.1.1. Dopamine Neuronal Activity

Dopamine neuron activity is crucial to proper brain function, and dysregulation of the dopamine system is involved in several pathologies (3). These neurons either fire in a pacemaker-like fashion (4), or burst fire in response to excitatory input (2). Pacemaking and burst activity of the dopamine neurons thereby contribute respectively to a tonic and phasic release of dopamine (5). This release (6-8) is calcium-dependent and occurs both within the projecting regions by the neuronal terminals (9-11) and locally in the midbrain through the somatodendritic dopamine release (6-8, 12) (see Figure 1).

Both pacemaking and burst firing properties of dopamine neurons are established by complex intrinsic ion conductances (2, 13). The main players in the pacemaking maintenance are the voltage-gated L-type calcium channels that induce near-threshold depolarization (14-16). Increased intracellular calcium activates subsequently small-conductance, calcium-activated potassium (SK) channels that hyperpolarize the cell (17-21). Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are activated in response, resulting in an inward hyperpolarization-activated cation current (I_h) (22). This leads to repolarization of the cell towards the threshold, reactivating the L-type calcium channels and causing the firing oscillation (23) at a rate of 1–5 Hz (7, 24).

In burst firing, the same ion channels are involved, but require an additional activation of the N-methyl-D-aspartate (NMDA) receptor, which is essential for the high calcium influx during burst firing (25, 26). NMDA receptors are ligand-gated ion channels with a high calcium permeability (27), which require binding of their ligand glutamate and obligatory co-agonist glycine or D-serine (28). Activation of the NMDA receptor is additionally subjected to voltage dependency. At low membrane potentials, magnesium will enter and bind the NMDA receptor pore, subsequently preventing the permeation of calcium ions (29, 30). Due to a depolarization of sufficient magnitude and duration, the magnesium ions are released from the pore, which allows an inward calcium flux (30). Together with the spontaneous depolarizing steps of the pacemaking dopamine neurons, glutamate activates the NMDA receptor, which gives rise to increased inward calcium currents. Consequently, the NMDA receptor contributes to the calcium-induced depolarization in the oscillating currents, as described earlier for pacemaking activity, but is rapidly blocked by magnesium in the hyperpolarization phase. Therefore, activation of the NMDA receptor allows the increased firing of >10 Hz in dopamine neurons called bursts (13, 31). The pauses which can be seen after bursts are mediated by the release of intracellular calcium stores through the activation of metabotropic glutamate receptors. Calcium thereby activates the SK channels resulting in a membrane hyperpolarization, which counteracts the oscillating currents during bursts (32).

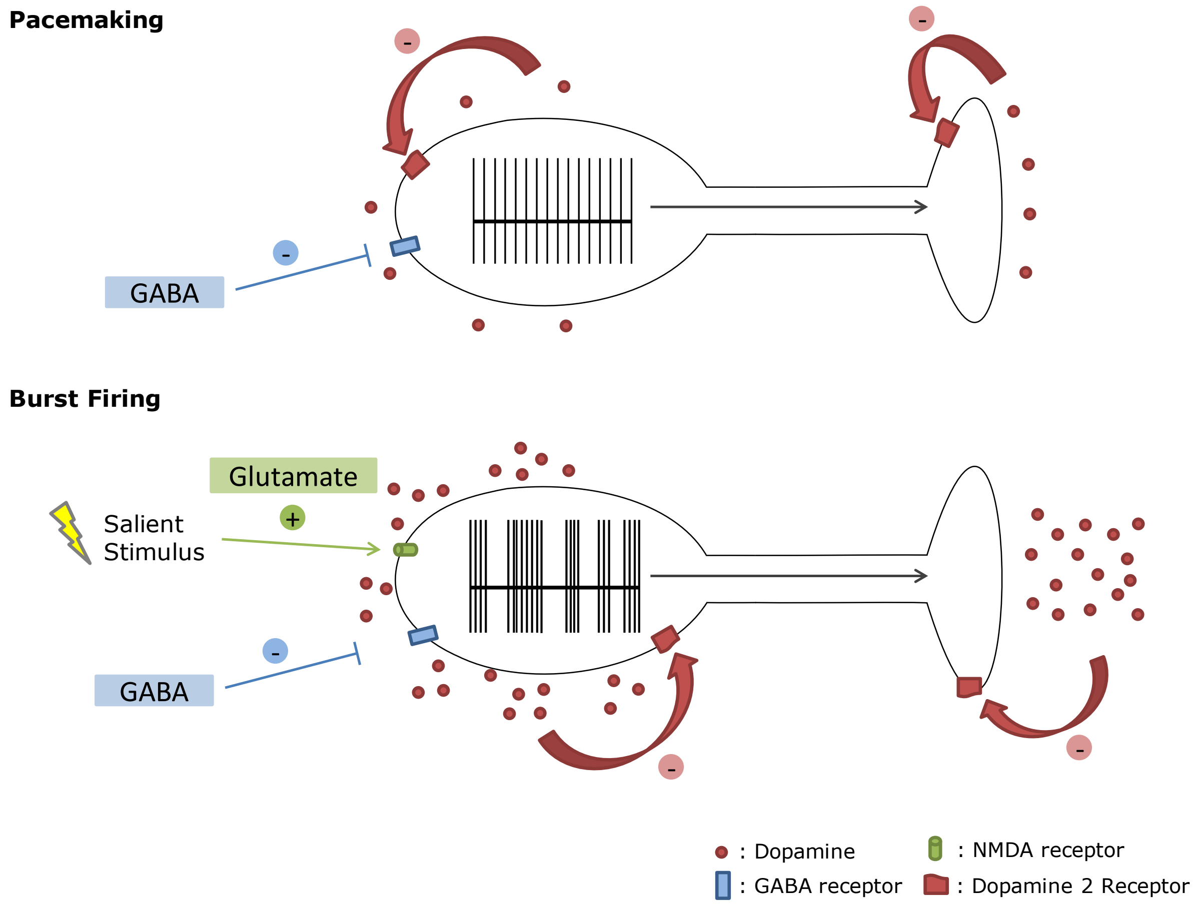

Figure 1: Midbrain dopamine neuron activity and modulation. Basal pacemaking activity of dopamine neurons induces a tonic release of dopamine at the somatodendritic site and terminals. NMDA release into the midbrain areas after a salient stimulus activate NMDA receptors on the dopamine neurons and induce burst firing. Burst firing subsequently causes phasic dopamine release at both the somatodendritic site and terminals. Dopamine release is self-inhibiting at the sites of release via activation of D2 dopamine autoreceptors. Additionally, afferent projecting and local GABAergic inputs inhibit dopamine activity.

1.1.2. Modulation of Dopamine Neurons

The activity of the dopamine neurons is tightly modulated by different inputs of neurotransmitters (see Figure 1). As mentioned earlier, the dopamine neurons are under control of glutamate release: NMDA receptor activation induces the onset of burst firing (31) and activation of metabotropic glutamate receptors also effectuate in the pauses seen after bursts (32). Glutamatergic afferents are provided from diverse brain areas both cortical (33-35) and subcortical (33, 36-40), which allows the integration of various environmental inputs, such as visual and auditory cues (39, 41). These glutamatergic inputs are triggered in response to novel, unexpected or salient events (2).

Though glutamtate is a very important regulator of the activity of dopamine neurons, γ-aminobutyric acid (GABA) synapses are the most prominent onto these neurons (42-48). Afferent GABAergic projections from the striatum, ventral and dorsal pallidum innervate dopamine neurons (42-46, 49), but also local GABAergic neurons within the midbrain attribute to GABAergic inputs (47, 50-52). GABA is an inhibitory neurotransmitter which can diminish the activity of dopamine neurons upon binding of its receptors (53-55). Two different types of GABA receptors mediate this inhibitory function (47, 50, 56). $GABA_A$ receptors are ionotropic chloride channels which induce inhibitory postsynaptic currents in the dopamine neurons upon activation (47, 52). $GABA_B$ receptors are metabotropic (57) and activate G protein-coupled inwardly-rectifying potassium channels (GIRK). GABA-induced hyperpolarization causes inhibition of dopamine neuron activity and suppresses burst firing (58, 59). Additionally, removal of GABAergic inhibition can contribute to the onset of burst activity (47, 60, 61).

Dopamine itself can furthermore inhibit dopamine neuron activity via activation of D2 dopamine autoreceptors on their cell bodies and dendrites. Activation of this metabotropic receptor activates GIRK channels and causes hyperpolarization (62-66). In this way, somatodendritic dopamine release modulates activity amongst neighboring dopamine neurons via dendrodendritic synapses (7, 67-69). At the terminal site, dopamine release inhibits the activity in a similar way, through activation of D2 autoreceptors present at the terminals (70). Therefore, the dopamine release has an autoregulating effect.

1.2. Glycine Receptor

Modulation of dopamine neuron activity tightly regulates dopamine release, and can therefore be an interesting therapeutic target. Another important neurotransmitter which can establish such modulation is glycine (71), yet the distinct roles of different glycine receptors (GlyRs) on dopamine neuron activity has not been studied. GlyRs are ionotropic chloride channels which are part of the ligand-gated nicotinic acetylcholine receptor family, also including the $GABA_AR$ and $GABA_cR$. Consequently, these receptors show a homologous structure (Figure 1) and functionality (72).

1.2.1. Structure and Function

GlyRs are transmembrane protein complexes consisting of five subunits organized around a central ion pore. Five different subunit isoforms are identified thus far: α1–4 and β (72). The subunits can either form homopentamers composed of a single α-subunit or heteropentamers formed by 2 α- and 3 β-subunits (73). Each subunit contains a large extracellular N-terminus, four α-helical transmembrane domains (TM 1-4), an intracellular loop, and a small extracellular C-terminus. The extracellular N-terminus contains the ligand-binding site of the receptor and has a cysteine loop incorporated, a characteristic of the ligand-gated inhibitory channels. The GlyRs can be activated by several agonists on its ligand-binding site , though with different potencies: glycine > β-alanine > taurine > L-serine. The transmembrane domains TM1, TM3 and TM4 act as an interface for the lipid bilayer integration of TM2, which is oriented towards the ion pore and controls the ion selectivity. The function of the intracellular loop linking TM3 and TM4 is the mediation of a variety of GlyR interactions, like phosphorylation and protein-protein interactions (72).

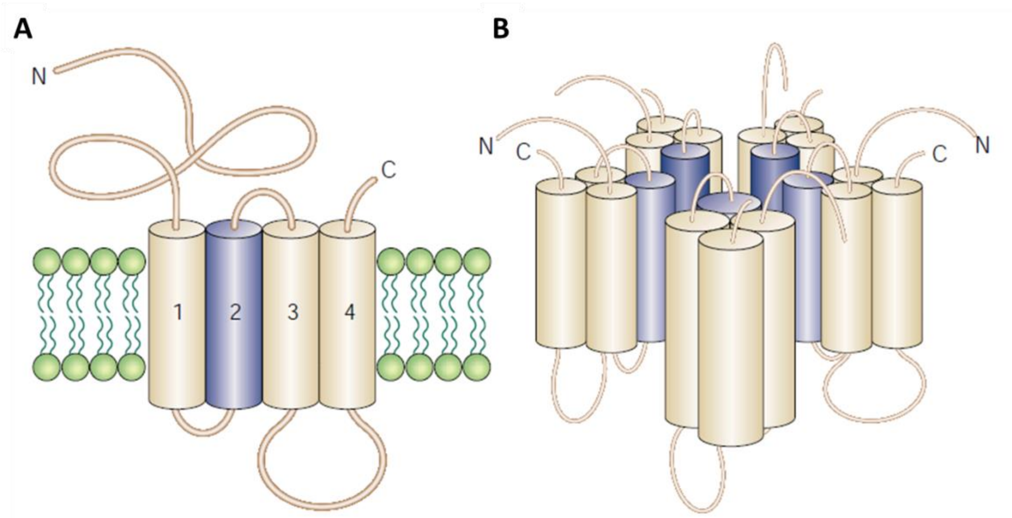

Figure 2: Structure of glycine receptors. A: The glycine receptor subunits comprehend a large extracellular N-terminus, four α-helical transmembrane domains (TM 1-4), a intracellular loop, and a small extracellular C-terminus. **B:** Glycine receptors are pentameric chloride channels. The central pore is aligned by the TM2 of each subunit. Figure adjusted from Moss and Smart, 2001 (74).

The α2-subunit is the predominant subunit in the embryonic and early postnatal brain and is thought to be expressed extrasynaptically as a homopentamer (75, 76). During maturation, a switch from homomeric GlyRα2 expression to synaptic heteromeric GlyRα1β-receptors occurs (73). Although, functional expression of the GlyRα2 was revealed in adult hippocampus, cerebral cortex and striatum (77-79), indicating that the initially proposed switch of subunit expression is not complete. Moreover, it appears that the GlyRα2 is the only functional glycine receptor present in adult striatum (internal communication with collaborative lab). GlyRα3s are not expressed at the embryonic stages, but also develops during maturation, like the GlyRα1s (73, 75, 76). The α4-subunit is expressed in the embryonic brain at low quantities followed by a further decrease in expression throughout development (73). So far, the α4-subunit was only identified in mice (80), zebrafish (81) and chicks (82), but not in humans. The β-subunit makes part of the heteromeric GlyRs and using its intracellular loop, the β-subunit can bind gepherin. Gepherin on its turn binds the cytoskeleton, which clusters, accumulates and stabilizes the heteromeric receptors at postsynaptic sites (72, 73).

Activation of the GlyRs induces a chloride flux moving the membrane potential towards the equilibrium potential of chloride (72). The embryonic and early postnatal brain show raised intracellular chloride concentrations contributing to a higher equilibrium potential. Consequently, the activation of the GlyRs results in cell depolarization. In mature neuronal cells it has an opposite effect: the intracellular chloride concentration is shifted to lower concentrations due to the expression of potassium-chloride cotransporter 2 (83). This concentration shift results in a decrease of equilibrium potential close to or more negative than the resting potential of the cell. Therefore, GlyRs fulfil an inhibitory function in mature neurons.

1.2.2. Role of Glycine Receptors in the Midbrain

In the adult brain, glycine can modulate midbrain dopamine neurons in different ways. Activation of GlyRs on dopamine neurons directly inhibits their activity (84, 85). However, GlyRs are also expressed on GABAergic neurons in the midbrain and produce opposite effects. Activation of GlyRs decreases GABAergic inhibition of dopamine neurons (49, 86), thereby enhancing the excitability of dopamine neurons (see Figure 3).

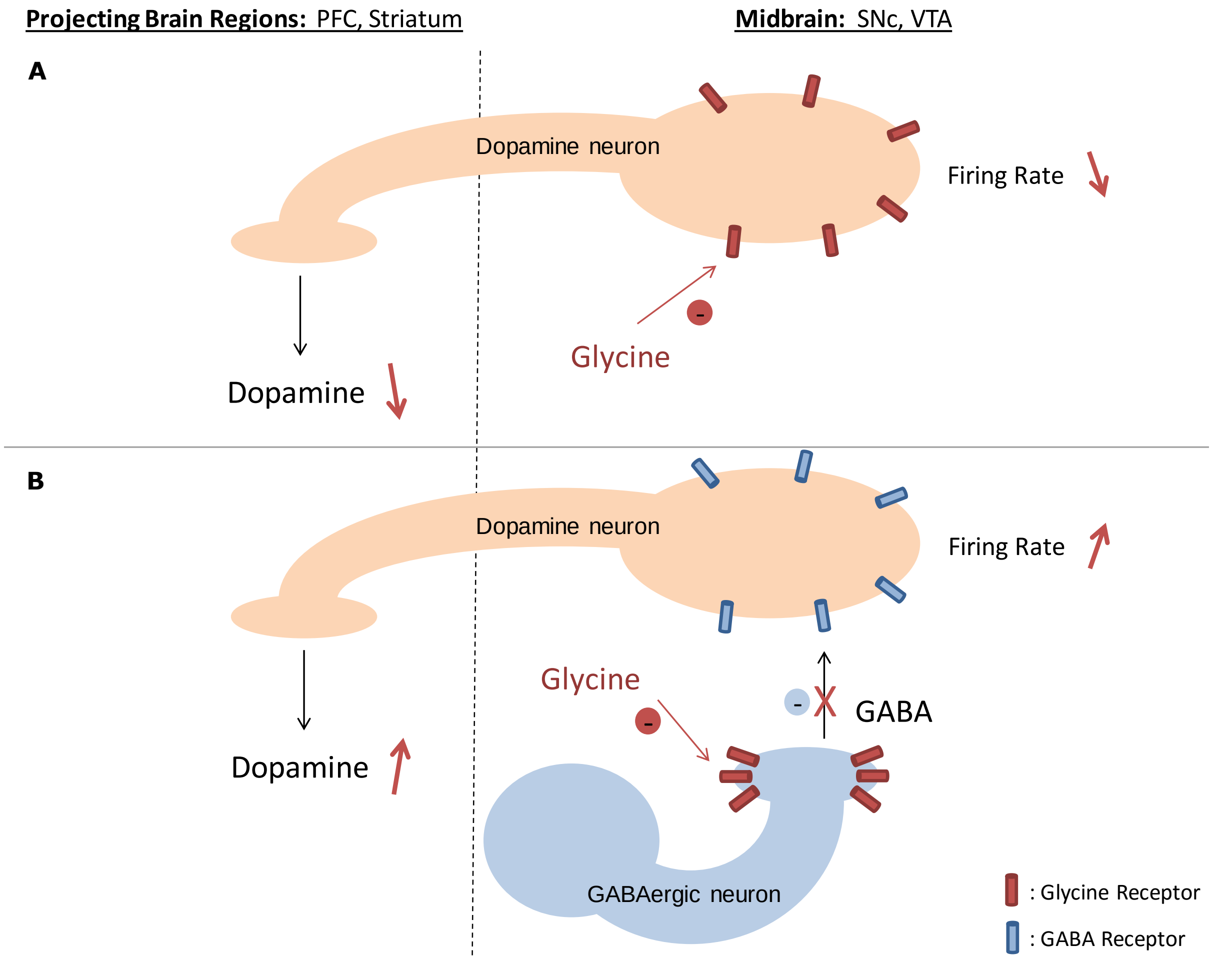

Figure 3: Glycine as a modulator of dopamine neurons. A: Activation of glycine receptors present on dopamine neurons directly inhibit activity and subsequently cause a decrease in dopamine release. **B:** Activation of glycine receptors present on GABAergic terminals inhibit GABA release and reduces activation its receptors on dopamine neurons. Reduced activation of inhibitory GABA receptors enhance excitability of dopamine neurons and thereby stimulate dopamine release.

Thus, glycine plays a major role in the modulation of dopamine neuron activity, yet, it is unclear which subunits are involved. However, preliminary research in mice revealed GlyRα2s as potential modulators in dopaminergic signaling. GlyRα2 knock-out (GlyRα2KO) mice showed greater activity after amphetamine treatment compared to wild-type littermates, which reflects an upregulated activity within the dopamine system. Up until now, little is known about GlyRα2s in the midbrain and they are thought to be present only as homomeric receptors which undergo a maturation switch (87). In the striatum they are involved intrinsic firing properties and density of corticostriatal projecting neurons (77). In contrast to the function of GlyRα2s in the adult midbrain, the role of these receptors has already been examined extensively during brain development. At embryonic stages, GlyRα2 activation in the cortex controls neurogenesis (88), tangential migration of interneurons (89), and GlyRα2 deficiencies display morphological and synaptic defects within the cortex. These findings indicate the importance of functional GlyRα2s in the cortical circuitry formation (90).

However their function in the brain development as homomeric receptors is well described, the presence of functional heteromeric GlyRα2s in the adult midbrain is yet to be investigated. In this study we hypothesized that glycine modulates dopamine neuron activity via activation of GlyRα2s. Therefore, we first aimed to confirm this direct effect of glycine on basal dopamine neuron activity in the SNc. Next, we investigated which GlyR subunits could effectuate this direct modulation of dopamine neurons in the midbrain. Finally, in a first step towards elucidating distinct roles of the GlyRs subtypes, we focused on the GlyRα2 subunit based on preliminary data and availability of a GlyRα2KO mouse model.

2. Materials and Methods

1.3. Animals

Animal experiments were performed according to the guidelines of the local ethical committee at Hasselt University. Male adult C57BL/6J wild-type (WT) mice and their GlyRα2KO (89) littermates were used with a minimum age of 42 days. Genotyping was performed using the KAPA Mouse Genotyping Kit (Kapa Biosystems, Belgium) and the following primers (respectively forward and reverse): 21: 5′-TGATCCTTTTCTGCTTCCAG-3′ and 5′-AATGTTGCAAACACCACCGA-3′; Ex: 5′-CACATGAACCCCAACACAAG-3′ and 5′-GCTTTTCGACAAGACCTTTGG-3′ (data not shown). Electrophysiological experiments were performed blind and genotyping was done afterwards.

1.4. Electrophysiology

During the experiments an artificial cerebrospinal fluid (aCSF) bubbled with 95% O_2/5% CO_2 was used, containing (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 21.4 $NaHCO_3$, and 11.1 glucose. Brains of adult mice were isolated and mounted on a Leica VT1200S vibrating microtome (Leica, Belgium) in ice-cold aCSF containing 1.25 mM of a NMDA blocker, kynurenic acid (Sigma-Aldrich, Belgium). Horizontal slices of 200 µm were cut of the ventral mesencephalon containing the SNc. The collected slices were put in recovery for at least 0.5 h at 36 °C in aCSF containing 1.25 mM kynurenic acid. During the recordings slices were continuously perfused with normal aCSF at a flow rate of 1.5-2 ml/min and held at a temperature of 36 °C.

Dopamine neurons were identified visually, as large neurons close to the medial terminal nucleus of the accessory optic tract, and by their electrophysiological properties, pacemaking activity of 1-4 Hz. Loose cell-attached voltage clamp (0 mV) recordings were performed using a Heka EPC9 (Heka elektronik, Germany) amplifier. Pipettes with a 4-8 MΩ resistance were used for recording containing a sodium-HEPES-based buffer (plus 20 mM NaCl; 290 mOsm/L; pH 7.35–7.40) (14, 91). For determining the effects of glycine on midbrain dopamine neurons, pacemaking activity was recorded at baseline (4 min), 1 mM glycine application (6 min, bath perfused), and wash-out (10 min).

Data were acquired using the Patchmaster (Heka elektroniks) software. The firing rate was analyzed by Clampfit (Molecular Devices, United Kingdom).

1.5. RNA extraction and real-time PCR

The VTA together with SNc were dissected from WT and GlyRα2KO mice (n=6 per group), followed by storage at -80 °C in autoclaved phosphate-buffered saline (PBS) until RNA extraction. Total RNA isolation was performed using QIAzol Lysis Reagent (Qiagen, Netherlands) and chloroform extraction followed by RNeasy Kit (Qiagen) purification. The absorbance ratio (A_{260}/A_{280} ~ 2), determined by NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Belgium), was used as a measurement for RNA purity. The purified RNA, at the amount of 742 ng, was converted to single-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Netherlands) and diluted 1:10 in autoclaved ultrapure water. Real-time PCR with a comparative threshold cycle (Ct) quantitation was performed, using a StepOnePlus Real-Time PCR System

(Applied Biosystems) and Fast SYBR Green Master Mix (Applied Biosystems), in order to determine the expression of the GlyR subunits. The expression levels of the target genes were normalized to expression of housekeeping genes Pgk1 and Hprt. All reactions were performed in duplicate. The following primers were used in the real-time PCR (respectively forward and reverse): Glra1: 5′-GGA AGAGGCGACATCACAA-3′ and 5′-GTCCGGAGAGAGGATGTCCA-3′; Glra2: 5′-CACTGGCAAGTTTACCTG CAT-3′ and 5′-GGAGACCCAGGACAAAATGA-3′; Glra3: 5′-GCACTGGAGAAGTTTTACCG-3′ and 5′-GAC ACCATCTCCCGAGCCTGCTT-3′; Glra4: 5′-CAGCATCAGATTGACCCTCA-3′ and 5′GCAGGAGCATCTTCT AGCCA-3′; Glrb: 5′-CTGTTCATATCAGCACTTTGC-3′ and 5′-CTGTTCATATCAGCACTTTGC-3′; Pgk1: 5′-GAAGGGAAGGGAAAAGATGC-3′ and 5′-GCTATGGGCTCGGTGTGC-3′; Hprt: 5′-CTCATGGACTGATT ATGGACAGGAC-3′ and 5′-GCAGGTCAGCAAAGAACTTATAGCC-3′ (Integrated DNA Technologies, Incorporation, Belgium).

1.6. Immunohistochemistry

Adult WT mice were perfused transcardially with PBS and fixated with 4% paraformaldehyde (PFA). The brains were isolated, fixated further in 4% PFA overnight at 4 °C and cryoprotected in a 30% sucrose solution overnight at 4 °C. The brains were embedded in FSC 22 Frozen Section Media (Leica) and stored at -80 °C. Cryosections (20 μm) were made using a Leica CM3050 S cryostat (Leica). The cryosections were treated with 50 mM NH_4Cl for 30 minutes before staining to avoid aspecific binding and washed 3 times with PBS for 5 minutes. Blocking was performed for 1 hour using PBS containing 10% normal donkey serum (NDS) and 1% bovine serum albumin (BSA). Primary antibodies goat anti-GlyRα2, N18 (1:100, Santa Cruz Biotechnologies, Germany), and mouse anti-tyrosine hydroxylase (TH, 1:200, Santa Cruz Biotechnologies) diluted in PBS containing 3% NDS and 1% BSA were used. After overnight incubation at 4 °C, the cryosections were washed 3 times in PBS for 5 minutes. Secondary antibodies donkey anti-mouse labelled with Alexa 488 (1:500, Life Technologies) and donkey anti-mouse labeled with Alexa 555 (1:500, Life Technologies) were applied for 1 hour diluted in PBS containing 3% NDS and 1% BSA. A nuclear counterstaining was carried out using DAPI (1:100, Life Technologies) followed by a wash step in PBS of 3 times 10 minutes. Finally, the cryosections were mounted using Fluorescence Mounting Medium (Dako, Belgium) and observed under a Nikon Eclipse 90i fluorescent microscope (Nikon Instruments, Belgium).

1.7. Statistics

Differences in electrophysiological properties between WT and GlyRα2KO mice were analyzed using two-way repeated-measures ANOVA with a Bonferroni post-hoc test. The relative expression of GlyR subunits in WT and GlyRα2KO mice were analyzed using a Student`s t test.

3. Results

3.1. Glycinergic Inhibition of Pacemaking Activity in Dopamine Neurons

In order to confirm the direct effect of glycine on dopamine neuron pacemaking activity, loose cell-attached voltage-clamp measurements were carried out. The pacemaking activity of dopamine neurons residing in the SNc was measured at baseline and after 1 mM glycine application. The neurons showed a baseline pacemaking firing rate of 1.96 ± 0.30 Hz, which strongly decreased after glycine application to a mean of 0.35 ± 0.36 Hz and stopped firing completely in 5 out of the 7 cells ($p<0.0001$; see Figure 4). These results indicate the presence of GlyRs on dopaminergic cells in the SNc.

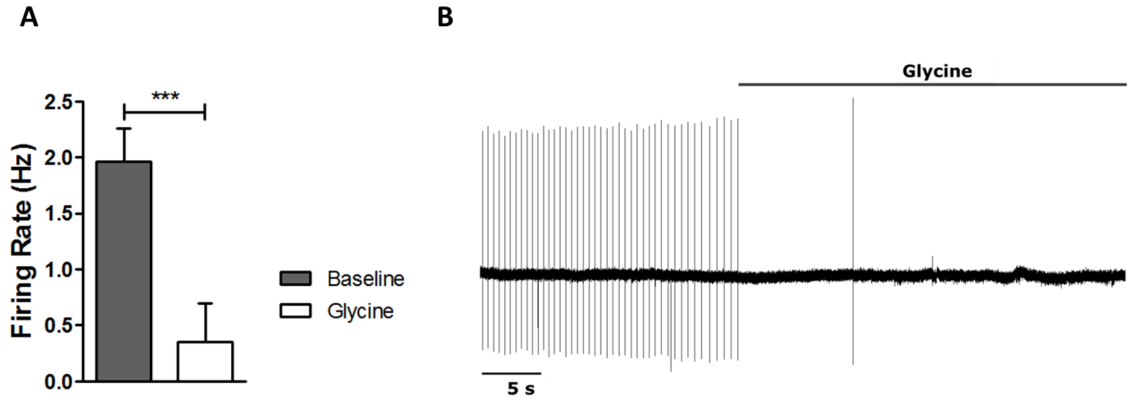

Figure 4: Effect of glycine on the pacemaking firing rate of dopaminergic neurons in the substantia nigra pars compacta of adult wild-type mice. A: Pacemaking firing rate of dopamine neurons at baseline and 1 mM glycine application. Results are given as mean ± SEM (n=7; *** $p<0.0001$). **B:** Representative trace of glycinergic inhibition of pacemaking activity in dopamine neurons.

3.2. Glycine Receptor Subunit Expression in the Midbrain

To further investigate the involved GlyR subunits, a real-time PCR was performed to indicate which subunits are expressed within the midbrain region (VTA and SNc) of adult WT mice. The real-time PCR results (n=6) showed the expression of all subunits (α1–4 and β), with respective Ct values of: 23.98 ± 0.14; 19.72 ± 0.12; 25.10 ± 0.17; 30.60 ± 0.18 and 20.02 ± 0.13 (see Figure 5A). Ct values below 30 represent an abundance of mRNA template and were measured for GlyRα1-3 and GlyRβ, while values between 30 and 35 indicate moderate amounts, as measured for GlyRα4. The primer efficiencies were not known, therefore calculation of the copy number and comparison between subunits was not possible. Though the expression of al subunits was revealed in the midbrain, it was not specific for dopamine neurons. Not only dopamine neurons attribute to these results, but all midbrain residing neurons, such as GABAergic neurons.

Based on preliminary data and the availability of the GlyRα2KO mouse model in the lab, the role of GlyRα2s in the modulation of pacemaking activity in dopamine neurons was put in focus. This as a first step to elucidate the distinct roles of the GlyR subtypes. To indicate the expression of GlyRα2 subunits on dopamine neurons in the SNc, an immunohistochemical co-staining of dopamine neurons (TH) and the GlyRα2 subunits (N18) was performed. The staining revealed the expression of GlyRα2 subunits by dopamine neurons as N18-fluorescent dots on TH-positive cells (see Figure 5B).

Before determining the role of GlyRα2s on modulating pacemaking activity of dopamine neurons, the expression of the subunits in GlyRα2KO mice was compared relatively to WT mice (n=6 for each group). This experiment was performed to ensure no involvement of the other GlyR subunits in possible functional differences between dopamine neurons of WT and GlyRα2KO mice. The GlyRα2KO mice showed a complete GlyRα2 knock-out (p<0.01) and no differences were found in expression of other GlyR subunits. The results are shown in Figure 5C.

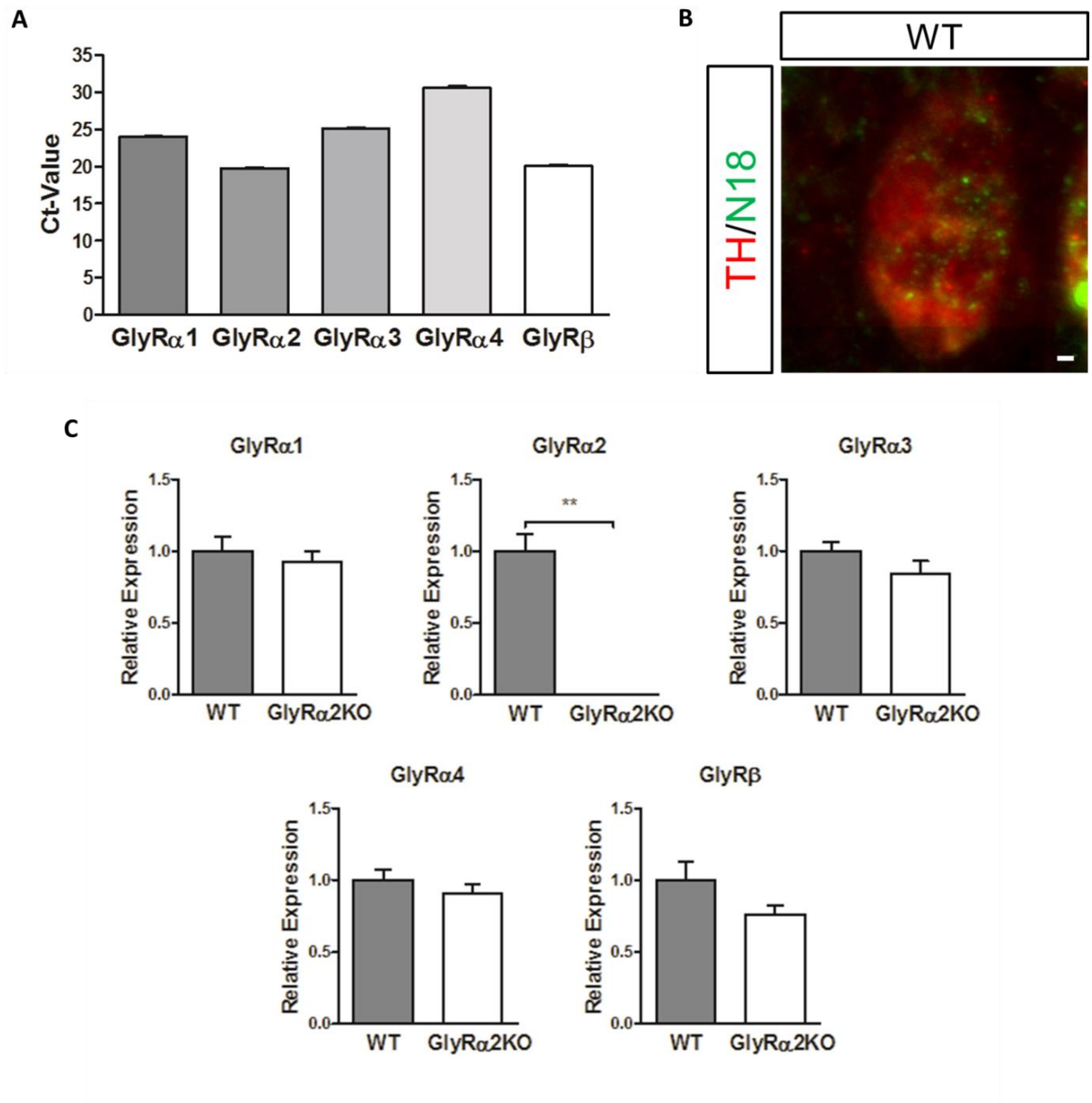

Figure 5: Expression of glycine receptor subunits in the midbrain. A: Threshold cycle (Ct) numbers of glycine receptor subunit mRNA in the midbrain. Values up to a 30 Results are given as mean ± SEM (n=6). **B:** Immunohistochemical co-staining of dopamine neurons (TH) and glycine receptor α2 subunits (N18) in the SNc. Scale bare: 1µm. **C:** Relative mRNA expression of glycine receptor subunits in the midbrain of WT (VTA, SNc). The GlyRα2KO mice show a complete GlyRα2 knock-out. No alterations in expression of other glycine receptor subunits were found between the GlyRα2KO and WT mice. Results are given as mean ± SEM (n=6 for each group; ** p<0.01).

3.3. Role of Glycine α2 Receptors in Pacemaking Activity of Dopamine Neurons

To determine the role of GlyRα2s, the electrophysiological measurements done in WT mice were repeated in the GlyRα2KO model. Dopamine neurons of the GlyRα2KO mice had firing rate of 1.87 ± 0.11 Hz at baseline activity and completely ceased fire after 1 mM glycine application (n=5, p<0.0001). These results were similar to the observations in WT mice, hence no differences in firing rate were detected (see Figure 6A and C). Next the coefficient of variation, which represents the irregularity of firing within a cell, was analyzed and compared between WT and GlyRα2KO mice. They showed an coefficient of variation of respectively 0.09 ± 0.02 and 0.11 ± 0.02 (see Figure 6B), which were not significantly different. Thus, the GlyRα2s did not have any observable modulatory effect on the pacemaking activity of dopamine neurons within these experiments.

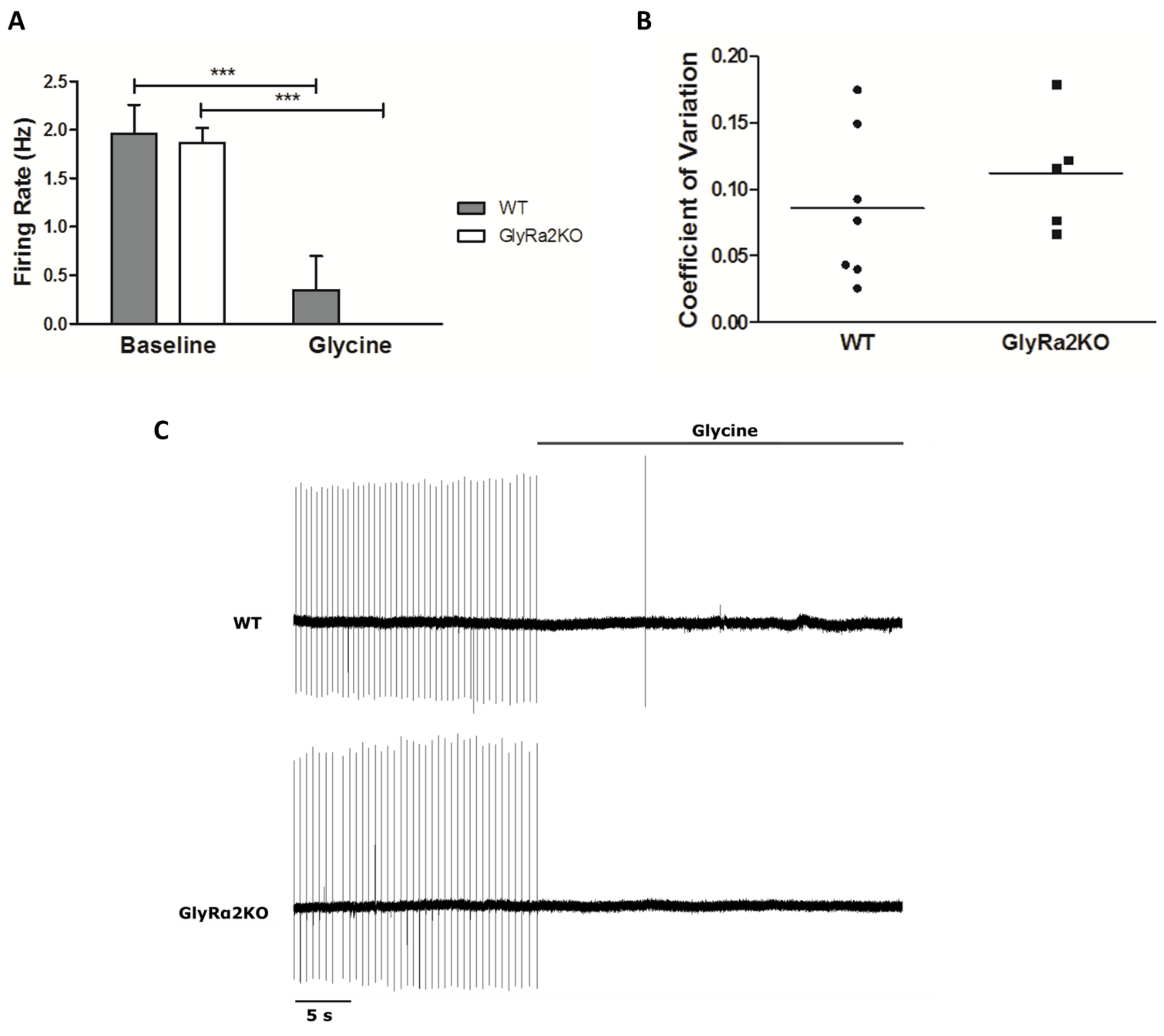

Figure 6: Comparing results of the glycinergic effect on pacemaking firing rate of dopaminergic neurons in the substantia nigra pars compacta of adult wild-type (WT) versus glycine receptor alpha 2 knock-out (GlyRα2KO) mice. A: Pacemaking firing rate of WT and GlyRα2KO dopamine neurons at baseline and 1 mM glycine application. Results are given as mean ± SEM (WT: n=7, GlyRα2KO: n=5 ; *** p<0.0001). **B:** Coefficient of variation of the baseline interspike interval within WT and GlyRα2KO dopamine neurons. Results are given as a scatter dot plot indicating the mean (WT: n=7, GlyRα2KO: n=5). **C:** Representative traces of glycinergic inhibition of pacemaking activity in WT and GlyRα2KO dopamine neurons.

4. Discussion

The present study aimed to confirm the direct effect of glycine on basal dopamine neuron activity in the SNc of the adult midbrain. Based on preliminary data, we further investigated the GlyRα2 subunit as a first step in elucidating the distinct roles of the GlyRs subtypes.

Loose cell-attached voltage-clamp measurements in brain slices of adult wild-type mice revealed the presence of inhibitory glycine receptors on dopamine neurons in the SNc. Application of 1 mM glycine significantly decreased pacemaking firing of dopamine neurons and even ceased firing completely in 5 out of the 7 cells. These results are in agreement with the previously described inhibitory effects of GlyR activation (84, 85). These original experiments were performed in whole-cell configuration, which influenced the recordings via alterations in ion concentrations by interaction of the pipet solution with the cytosol. Loose cell-attached measurements, as used in this study, prevent this influence and should be used instead to measure the activity (92).

It should be noted that we applied a concentration resemblant of synaptic concentrations (93). It is conceivable that glycine at these concentrations also inhibits burst firing. Burst firing causes the release of large amounts of dopamine in the projecting regions, such as the striatum. Here it contributes to events of psychosis (94-97), while on the other hand, it can also alleviate motor dysfunction in Parkinson's disease (2). Thus, the glycine-induced inhibition can be an interesting target for treatment with respectively antagonist and agonists. Future experiments will therefore determine the inhibiting potencies after glutamate-stimulated burst firing in dopamine neurons.

While it was earlier shown that tonic glycine concentrations can attenuate dopamine neuron activity by inhibition of neighboring GABAergic neurons (86), this effects is abolished by the direct activation of glycine receptors on dopamine neurons at synaptic resemblant concentrations. Therefore, we will investigate the modulation of dopamine neuron activity in basal conditions at tonic glycine concentrations, and differentiate between direct inhibitory (observed in this study) and indirect excitatory (86) effects of glycine. Firing rate experiments at glycine concentrations of 1-10 µM (93) are to be performed in the presence or absence of GABA blockers. These measurements will respectively give rise to the direct inhibitory and total effect of glycine on dopamine neurons. Subsequently, the indirect excitatory effects via the GABAergic neurons can be deduced.

In order to investigate which GlyR subunits contribute to the glycinergic inhibition, a real-time PCR was performed on mRNA extracted from midbrain tissue. This revealed the expression of all subunits within the midbrain. These results are, however, not specific for dopamine neurons, but reflect subunit expression on the general midbrain population. In a next step towards the investigation of GlyR subunits expressed by dopamine neurons, we focused on the GlyRα2 based on the preliminary data, which showed the involvement of the α2 subunit in dopaminergic signaling. An immunohistochemical staining of TH and GlyRα2s revealed the presence of the receptors in a dot-like manner on dopamine neurons. These findings are in line with the findings which indicate that the initially proposed switch of GlyRα2s to GlyRα1βs expression during maturation is not complete (77-79). Additionally, the dot-like presence of the GlyRα2s on dopamine neurons suggests the expression of synaptic clustered heteromeric receptors. This also contradicts with the initial postulation that GlyRα2 are solely expressed as extrasynaptic homopentamers (75, 76, 87). A co-staining for dopamine neurons, GlyRα2 and gepherin, which binds the β-subunit and clusters heteromeric GlyRs at postsynaptic sites, will confirm the expression of heteromeric GlyRα2s on dopamine neurons (72, 73).

The next step was to determine the modulatory role of GlyRα2s on basal dopamine neuron activity and for this a GlyRα2KO mice model was used. Yet, nothing was known about effects of the knock-out on other GlyR subunits expressed by dopamine neurons. To ensure no involvement of GlyR subunits other than GlyRα2 in possible functional differences between WT and GlyRα2KO dopamine neurons, the relative expression was checked for all subunits. GlyRα2 showed a complete knock-out in GlyRα2KO mice, while expression of the other subunits was not affected. Therefore, functional differences of dopamine neurons between WT and GlyRα2KO littermates could be attributed to the modulatory role of GlyRα2s.

Electrophysiological observations made in GlyRα2KO dopamine neurons were similar to those of WT cells. This indicates contribution of other GlyR subunits in the complete inhibition of pacemaking firing at this glycine concentration. Although there is no noticable involvement of GlyRα2s at baseline pacemaking activity or after 1 mM glycine application, GlyRα2s can still have modulatory effects at lower tonic glycine concentrations and/or in burst firing modulation. Still, one should notice possible compensatory and/or interacting mechanisms occurring during the brain development or in the adult brain. To be sure to avoid these mechanisms, a conditional knock-out model specific for GlyRα2 should be used, yet, this is just recently commercially available.

5. Conclusion

This study confirmed that activation of GlyRs on midbrain dopamine neurons fulfils an inhibitory role on pacemaking activity. In a first step towards unveiling the roles of the different subunits, we revealed the presence of GlyRα2 on dopamine neurons, yet electrophysiological measurements showed no modulatory effects of GlyRα2 on baseline dopamine neuron activity or in response to glycine application at synaptic concentrations. However, it is conceivable that GlyRα2s play a modulatory role at lower tonic glycine concentrations and/or in burst firing. These findings can contribute to better insights into the etiology of dopamine-related diseases, such as schizophrenia and drug abuse, and development of new treatment strategies. Elucidating the distinct roles of different subunits on dopamine neuron activity in the future will contribute even more.

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Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
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Jaar: **2016**

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Voor akkoord,

Devoght, Jens

Datum: **8/06/2016**