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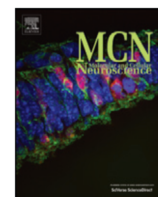

Methyl-4-phenylpyridinium (MPP+) differentially affects monoamine release and re-uptake in murine embryonic stem cell-derived dopaminergic and serotonergic neurons

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1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) is known to selectively damage dopaminergic (DA) cells in the substantia nigra and to produce symptoms which are alike to those observed in Parkinson's disease (PD). Based on the similarity between MPTP-induced neurotoxicity and PD-related neuropathology, application of MPTP or its metabolite methyl-4-phenylpyridinium (MPP+) was successfully established in experimental rodent models to study PD-related neurodegenerative events. MPP+ is taken up by the dopamine transporter (DAT) into DA neurons where it exerts its neurotoxic action on mitochondria by affecting complex I of the respiratory chain. MPP+ is also a high affinity substrate for the serotonin transporter (SERT), however little is known about possible toxic effects of MPP+ on serotonergic (5-HT) neurons. In order to compare cell type-specific effects of MPP+ treatment, we have differentiated mouse embryonic stem (ES) cells into DA and 5-HT neurons and studied the impact of MPP+ treatment on both types of monoaminergic neurons in vitro. MPP+ treatment impacts on mitochondrial membrane potential in DA as well as 5-HT ES cell-derived neurons. Although mitochondria metabolisms are similarly affected, synaptic vesicle cycling is only impaired in DA ES cell-derived neurons. Most importantly we show that MPP+ induces DAT externalization in DA neurons, but internalization of SERT in 5-HT neurons. This diverse MPP+-induced transporter trafficking is reflected by elevated substrate uptake in DA neurons, and diminished substrate uptake in 5-HT neurons. In summary, our experimental data point toward differential effects of MPP+ intoxication on neurotransmitter release and re-uptake in different types of monoaminergic neurons.

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1. Introduction

Parkinson's disease (PD) originally results from cell death of dopaminergic neurons in the substantia nigra pars compacta projecting into the striatum. This degeneration leads to loss of dopamine (DA) in the dorsolateral putamen in the striatum known to be causative for the typical motor symptoms of PD such as tremor, rigidity, bradykinesia and postural instability (reviewed in Dauer and Przedborski, 2003). Moreover, several non-motor symptoms such as chronic fatigue, impulse-control disorders, sleep disorders and depression are frequent within PD and contribute significantly to the disability of the patients as the disease progresses. These findings are indicative for a serotonergic dysfunction in PD in addition to a loss of DA neurons (Huot et al., 2011; Politis and Loane, 2011; Politis and Niccolini, 2015). Here, according to the Braak staging of PD, serotonergic neurons originating from the raphe nuclei become affected in stage 2 and 3 of the disease (Braak et al., 2003). As a consequence serotonin (5-hydroxytryptamine, 5-HT) levels should be decreased based on neurodegenerative events in the raphe nuclei at these disease stages. Interestingly, evaluation of patients revealed that, in contrast to the robust DA reduction observed in the putamen of all patients, patients' 5-HT levels were inconsistent, with reports of diminished 5-HT levels in several brain regions, including the substantia nigra (Huot et al., 2011), as well as 5-HT concentrations well within the range of healthy controls (Kish et al., 2008; Shannak et al., 1994).

In 1983 Langston and colleagues reported that four persons developed marked Parkinsonism after using 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) intravenously. On the basis of the striking parkinsonian features observed, and additional pathological data from one previously reported case, it was initially proposed and later shown that MPTP treatment selectively damages cells in the substantia nigra (Langston et al., 1983; Burns et al., 1983; Moratalla et al., 1992). The PD-like phenotype of MPTP treatment is caused by MPTP being

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metabolized into methyl-4-phenylpyridinium (MPP+), which is taken up into DA neurons by the dopamine transporter (DAT). Inside DA neurons MPP+ can be accumulated into synaptic vesicles by the vesicular monoamine transporter VMAT2 or into mitochondria. Here it exerts its neurotoxic action by targeting NADH dehydrogenase in complex I of the respiratory chain and thus lowers ATP production (Singer and Ramsay, 1990). Lower levels of ATP can elevate cytosolic reactive oxygen species (ROS) production by triggering DA efflux from synaptic vesicles into the cytoplasm due to the fact that VMAT2 can no more maintain the concentration gradient of DA inside the vesicles (Johnson, 1988). Although the clinical phenotype of PD is fairly homogenous, featuring disrupted DA neurotransmission in the substantia nigra as the main hallmark, the etiology of the PD phenotype is multifactorial. For example, regarding PD genotypes, several genes are associated with PD, including PARK mutations, of which one gene encodes for alpha-synuclein. Alpha-synuclein accumulates in so called Lewy bodies, abnormal protein aggregates that develop during the onset of PD. Further pathomechanisms shown to play key roles in PD pathogenesis include mitochondrial dysfunction, neuroinflammation and free radical-induced damage. Despite being considered as one of the major pathomechanisms underlying PD, the relationship of oxidative stress and mitochondrial dysfunction observed in PD is not well understood to date (Blesa et al., 2015; Kim et al., 2015; Schapira, 2008).

MPP+ also is a substrate for the serotonin transporter (SERT), and while MPTP or MPP+ treatment results in a robust decrease in striatal DA throughout all studies, 5-HT concentrations were found to vary significantly (Gorton et al., 2010; Maiti et al., 2016; Nayyar et al., 2009; Vuckovic et al., 2008), raising the question whether 5-HT neurons are less susceptible for MPP+ poisoning. Therefore, we aimed to identify possible cellular mechanisms how MPP+ exerts its effects on DA and 5-HT neurons *in vitro*. To this end we have differentiated DA and 5-HT neurons from mouse embryonic stem cells and studied the impact of MPP+ treatment on mitochondrial membrane potential and synaptic vesicle recycling as a correlate for neuronal functioning. Moreover, we have quantified the effect of MPP+ treatment on DAT and SERT cell surface density by antibody detection combined with confocal laser scanning microscopy (Lau et al., 2009; Matthaus et al., 2016), and uptake measurements of a fluorescent transporter substrate (Lau et al., 2015). Our findings provide evidence, that MPP+ treatment exerts different effects on stem cell-derived dopaminergic and serotonergic neurons *in vitro*.

2. Material and methods

2.1. Differentiation of mouse embryonic stem cells

Mouse embryonic stem (ES) cells were derived from a mouse strain [C57 BL/6N] that express the hSERT instead of the mSERT (Matthaus et al., 2016). ES cells were differentiated into midbrain dopaminergic neurons according to established protocols (Baizabal and Covarrubias, 2009; Lee et al., 2000; Kawasaki et al., 2000; Lau et al., 2006) and into serotonergic neurons as previously described (Matthaus et al., 2016). *In vitro* differentiation of ES cell started with the generation of neuronal precursor cells. During this stage, ES cells were transferred into neuronal stem spheres (NSS) medium (KO DMEM) containing 15% FBS ES quality, 2 mM L-glutamine (L-Gln, Life Technologies, Germany), 100 mM non-essential amino acids (NEAA, Life Technologies), 100 μM penicillin/streptomycin (Life Technologies), and supplemented with 20 ng/mL epidermal growth factor (EGF, #130-093-825), 25 ng/mL sonic hedgehog protein (SHH, #130-095-723), and 20 ng/mL fibroblast growth factor 8b (FGF8b, #130-095-731; all growth factors from Miltenyi Biotech, Germany) and 0.2 μg/mL ascorbic acid (#A-0278, Sigma Aldrich, Germany) for the dopaminergic differentiation or EGF, FGF8b, basic fibroblast growth factor (FGFb, #130-104-925, all 20 ng/mL), and 30 nM ethanolamine for the serotonergic differentiation (Matthaus et al., 2016). NSS formation required 3 to 4 days. After this stage, NSS were plated to laminin-gelatine-coated dishes (gelatine: 0.1% in PBS, #G7765, laminin 1 μg/mL, #L6274, both Sigma-Aldrich). For selection of dopaminergic precursors 10^6 cells per cm^2 and for selection of serotonergic precursors 10^5 cells per cm^2 were plated. Cells were kept in lineage selection medium (LS) for at least 4 days (DMEM/F12 GlutaMAX (#31331-093, Life technologies)). For dopaminergic differentiation LS medium was supplemented with 100 mM NEAA, 2 mM L-Gln, 100 μM Pen/Strep, 2% B27-supplement (#17504-044, Life Technologies), 1% N2-supplement (#17502-048, Life Technologies) and 20 ng/mL EGF, FGF8b, 25 ng/mL SHH and 0.2 μg/mL ascorbic acid (Lee et al., 2000; Kawasaki et al., 2000). For serotonergic differentiation, LS medium was supplemented with 20 ng/mL EGF, FGF8b and FGFb, and 30 nM ethanolamine (Matthaus et al., 2016). LS medium was changed every second day. Terminal neuronal differentiation (ND) was induced by removal of growth factors; ND medium consisted of DMEM/F12 GlutaMAX supplemented with 100 mM NEAA, 2 mM L-Gln, 100 μM Pen/Strep, 2% B27-supplement, 1% N2-supplement, and 10 ng/mL brain derived neurotrophic growth factor (BDNF, #130-096-286, Miltenyi Biotech). For dopaminergic differentiation ND medium was supplemented with 0.2 μg/mL ascorbic acid, for serotonergic differentiation with 30 nM ethanolamine. ND medium was exchanged every second day. Terminal differentiation was accomplished 14 days after start of differentiation and yielded mature ES cell-derived dopaminergic (ESN^{DA}) or serotonergic ($ESN^{5\text{-}HT}$) neurons.

2.2. MPP+ treatment and mitochondrial membrane potential imaging of MPP+ treated ESN^{DA} and $ESN^{5\text{-}HT}$

ESN^{DA} and $ESN^{5\text{-}HT}$ were treated for up to 5 h with 50 μM methyl-4-phenylpyridinium (MPP+; #D048, Sigma Aldrich), which was shown to significantly affect midbrain and striatal neurons *in vivo* and primary dopaminergic cultures *in vitro* (Choi et al., 2015). Mitochondrial membrane potential (MMP, $\Delta\Psi m$) of ESN^{DA} and $ESN^{5\text{-}HT}$ was monitored by acquisition of tetramethylrhodamine ethyl ester (TMRE) fluorescence (#T669, Thermo Scientific, Germany) in live cell imaging experiments performed with a Leica TCS SP5 imaging system attached to a DM IRE2 microscope. TMRE is a fluorescent probe, which equilibrates rapidly across the mitochondrial membranes without almost any association with other organelles. ESN^{DA} and $ESN^{5\text{-}HT}$ were loaded with 200 nM TMRE for 20 min. To quantify the effect of MPP+ treatment on $\Delta\Psi m$, TMRE fluorescence images were acquired before the application of MPP+, to determine TMRE control fluorescence intensities. The laser settings applied to acquire control images, optimized to avoid over excitation of pixels and allow quantitative image recording, were kept consistent throughout image acquisition. After addition of MPP+ to the culture medium, images were acquired at the given time points from at least 5 regions of interest (ROI; $n = 25$ per time point). For the duration of treatment, culture dishes (Ibidi, Germany) were kept in the incubator and moved to the microscope stage for image acquisition. TMRE fluorescence intensities were quantified using ImageJ software (NIH, USA) after cutting off background fluorescence using a threshold value. At least 75 images of neurons from three independent experiments were recorded per control and treatment time point.

2.3. FM4-46 FX staining of active presynapses in MPP+ treated ESN^{DA} and $ESN^{5\text{-}HT}$

FM4-64FX staining was performed to identify neurotransmitter releasing synaptic vesicles of 50 μM MPP+ treated ESN^{DA} and $ESN^{5\text{-}HT}$ upon high potassium-induced depolarization. For each experiment, ES cell-derived neurons were processed as follows: MPP+ treated *ESN^{DA} and $ESN^{5\text{-}HT}$* were initially incubated in media containing 1 μM FM4-64FX (#F34653, Life Technologies) for 5 min at 37 °C. Afterwards, cells were incubated for 5 min at 37 °C in reaction buffer (60 mM KCl, 120 mM NaCl, 10 mM glucose, 10 mM HEPES, 4 mM CaCl2, pH 7.4) to induce neurotransmitter release (Lau et al., 2010). Control neurons were kept in potassium-free buffer (120 mM NaCl, 10 mM glucose, 10 mM HEPES, pH 7.4). Thereafter cells were incubated with media

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