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Rotenone Induces Cell Death of Cholinergic Neurons in an Organotypic Co-Culture Brain Slice Model

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Abstract

In Alzheimer and Parkinson's disease cell death of cholinergic and dopaminergic neurons are characteristic hallmarks, respectively. It is well established that rotenone, an inhibitor of complex I of the electron transport chain, induces cell death of dopaminergic neurons, however, not much is known on the effects of rotenone on cholinergic neurons. The aim of the present study was to evaluate the effects of rotenone on cholinergic neurons in an organotypic in vitro brain co-slice model. When co-cultures were treated with 10 μ M rotenone for 24 h a significantly decreased number of cholinergic neurons was found in the basal nucleus of Meynert but not in the dorsal striatum. This cell death exhibited apoptotic DAPI-positive malformed nuclei and enhanced TUNEL-positive cells. In summary, inhibition of complex I of the electron transport chain may play a role in neurodegeneration of cholinergic neurons.

Keywords

Rotenone; Cholinergic neurons; Inhibitor of complex I; In vitro model

Introduction

Neurodegeneration in several brain regions are the hallmarks of various brain disorders. Alzheimer's disease (AD) is a chronic progressive disease, accompanied by cell death of cholinergic neurons of the basal nucleus of Meynert (nBM) and septum. A lack of acetylcholine in the cortex and hippocampus directly correlates with cognitive decline and memory dysfunction [1–4]. Parkinson's disease (PD) is characterized by the degeneration of dopaminergic neurons of the ventral mesencephalon (vMes) and consequently, the lack of dopamine in the dorsal striatum (dStr) causes motor dysfunction in humans [5, 6]. The causes for cell death of dopaminergic neurons in PD and cholinergic neurons in AD are not known. Several cell death inducing stimuli are discussed, such as oxidative stress [7–10], glutamate-induced cell death [11, 12], toxic compounds [6, 13], pro-inflammatory cytokines [14, 15] or pro-apoptotic stimuli [16, 17]. Rotenone is one of these cell death inducing stimuli, which have been discussed to be involved in PD. Rotenone works as a classical, high affinity inhibitor of complex I of the electron transport chain (ETC), which leads to the

production of reactive oxygen species (ROS) and oxidative stress in neurons [18–22]. It has been assumed, that rotenone may be involved in the development of PD due to the reduced activity of complex I of the mitochondrial respiratory chain in the vMes [18].

The effect of rotenone on cholinergic neurons is not clear. Thus, the aim of the present work was to determine the effects of rotenone on the neuronal survival of cholinergic neurons in a well established four-slice co-culture model.

Experimental Procedure

Organotypic Brain Slice Cultures

Organotypic brain slice cultures were established as described by us in detail [23–26]. Briefly, for the co-culture system the basal nucleus of Meynert (nBM), ventral mesencephalon (vMes), parietal cortex (Ctx) and dorsal striatum (dStr) of postnatal day 7–9 (P7–9) Sprague Dawley rats was dissected under aseptic conditions, 400 μ m slices were cut with a tissue chopper (McIlwain, USA), and the four slices were placed (Fig. 1a) on a 30 mm diameter Millicell-CM 0.4 μ m membrane insert (Millipore, Austria), where they became attached to the membrane after 2 weeks of incubation. It is important to note, that the ipsilateral as well as contralateral brain areas were dissected at the same time, cut on the tissue chopper and all slices pooled in medium. Slices were cultured in 6-well plates (Greiner) at 37°C and 5% CO₂ with 1.2 ml/well of the following culture medium: 50% MEM/HEPES (Gibco), 25% heat inactivated horse serum (Gibco/Lifetech, Austria), 25% Hanks' solution (Gibco), 2 mM NaHCO₃ (Merck, Austria), 6.5 mg/ml glucose (Merck), 2 mM glutamine (Merck), pH 7.2. Medium was changed twice a week. Brain slice cultures were incubated with or without 10 ng/ml nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) for 4 weeks. To analyze the cell death of neurons, 4 weeks old cultures were incubated for 3 days without growth factors (growth factor withdrawal), treated afterwards with or without 10 μ M rotenone in medium for 24 h and then incubated for further 3 days in medium without growth factors. To test the appropriate rotenone doses, 2 week old nBM single slices were treated with 10 μ M, 1 μ M, 100 nM, 10 nM or 1 nM rotenone for 24 h. At the end of the experiment, slices were fixed for 3 h at 4°C in 4% paraformaldehyde/10 mM phosphate buffered saline (PBS) and then stored at 4°C in PBS until use. All experiments conformed to Austrian guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering.

Immunohistochemistry

Immunohistochemistry was performed as described previously [23]. All incubations were performed free floating for 2 days including 0.1% Triton, such that the antibodies can penetrate from both sides of the slices, which allows good penetration of the antibody into the brain slices. Slices were washed 30 min with 0.1% Triton/Phosphate-buffered saline (T-PBS) at room temperature and pre-treated 20 min with 20% methanol/1% H₂O₂/PBS (only for 3,3'-diaminobenzidine labeling). After thorough rinsing, the slices were blocked with 20% horse serum/0.2% BSA/T-PBS and then incubated for 2 days at 4°C with primary antibodies against choline acetyltransferase (ChAT, 1:750, Millipore), tyrosine hydroxylase

(TH, 1:500, Millipore) or dopamine D2 receptor (D2R, 1:250, Santa Cruz). Then the slices were washed again with PBS and incubated with secondary biotinylated anti-goat (ChAT), anti-rabbit (TH), or anti-mouse (D2R) antibodies (1:200, Vector Lab., USA) for 1 h at room temperature. Following further washing steps with PBS, slices were incubated in an avidin-biotin complex solution (ABC-Elite Vectastain reagent Vector Lab.) for 1 h. After being washed with 50 mM Tris-buffered saline (TBS), the signal was detected by using 0.5 mg/ml 3,3'-diaminobenzidine (DAB) including 0.003% H₂O₂ as a substrate in Tris-buffered saline. The slices were mounted on glass slides, air dried and coverslipped with Entellan (Merck, Germany). Unspecific staining was defined by omitting the primary antibody. For fluorescence immunohistochemistry the methanol pre-treatment was omitted and as secondary antibodies Alexa-488 (Invitrogen, 1:400) were used. To label nuclei, slices were incubated with 4,6-diamidino-2-phenylindole (DAPI, 1:10000; Sigma) for 30 min. Certain sections were stained with cresyl violet for 10 min and rinsed afterwards for further 5 min in aqua dest. Immunolabeling was visualized with a Leica DMIRB fluorescence inverse microscope equipped with an Apple computer.

DNA Nick-End Labeling (TUNEL Staining)

The TUNEL reaction was performed as described earlier [27]. Fresh co-slices were carefully transferred to glass slides, frozen on a CO₂ snow, and stored at -20°C until use. Co-slices were then thawed, and incubated with 150 U/ml terminal transferase (Roche) and 10 nmol/ml biotinylated 16-dUTP (Roche) in terminal transferase buffer (Roche) at 37°C for 2 h. Afterwards, co-slices were fixed with 4% paraformaldehyde/10 mM PBS for 30 min and then washed in PBS. Co-slices were incubated with ABC reagent (Vectastain) for 30 min at room temperature, then washed in 50 mM Tris buffer pH 7.6 (3 × 10 min) and the staining was performed in Tris pH 7.6 with 0.5 mg/ml DAB as a substrate, including 0.0003% H₂O₂ and 0.4% NiCl₂. Sections were extensively washed in aqua dest., air dried, and covered with Entellan.

Analysis, Quantification and Statistics

The number of microscopically detectable immunoreactive neurons was counted in the whole slice visualized under a 20× objective. The areas were identified by the respective immunohistochemical staining and by the mark placed at the top of the membrane insert (Fig. 1a). Cell counting was performed for DAPI-positive malformed and TUNEL-positive nuclei on a Leica DMIRB fluorescence inverse microscope equipped with an Apple computer and Improvision Openlab Darkroom software. Cell counting was performed on a random field (260 × 190 μm) per slice in the vMes, nBM and dStr. Multistatistical analysis was obtained by one way ANOVA, followed by Fisher PLSD posthoc test by comparing controls against the respective treatments, where $P < 0.05$ represents statistical significance.

Results

Morphology of Co-Slices Composed of Four Brain Regions

When co-cultures composed of four brain regions (nBM, cortex, vMes and dStr) were cultured for 4 weeks, the slices grew together and formed a large slice, which displayed several strong cresyl violet positive cells (Fig. 1b). Approximately 120 neurons per slice

(Table 2) of ChAT-positive neurons were found in the nBM slice (Fig. 1c), and approximately 50 neurons per slice (Table 2) were detected in the dStr slice (Fig. 1d). No ChAT-positive neurons were discovered in the cortex (Fig. 1e) or vMes (Fig. 1f). Approximately 80 TH-positive neurons per slice (Table 2) were found exclusively in the vMes (Table 2; Fig. 1g). Immunohistochemistry for D2R revealed several strongly stained cells in the dStr (Fig. 1h).

Effects of Rotenone in Co-Slices Composed of Four Brain Regions

When different doses of rotenone (1 nM–10 μ M) were applied onto single nBM slices, only the highest dose of 10 μ M induced cell death of cholinergic nBM neurons (Table 1). The number of ChAT-positive nBM neurons was also significantly decreased in the co-culture model after incubation with 10 μ M rotenone (Fig. 2 a–d), however, no effects were seen on cholinergic neurons in the dStr (Table 2). However, a reduced number of dopaminergic neurons was discovered after rotenone treatment in the vMes (Table 2). Some neurons exhibited a shrunken and smaller shape after rotenone treatment (Fig. 2b, d), compared to untreated co-slices (Fig. 2a, c). Furthermore, rotenone revealed a significantly increased number of TUNEL-positive nuclei (Fig. 2f; Table 2), compared to untreated co-cultures (Fig. 2e; Table 2). Likewise, rotenone enhanced the number of DAPI-positive malformed nuclei in the vMes, nBM and dStr (Table 2). Healthy nuclei appeared round, well-formed with an intensive staining (Fig. 2g), whereas, the malformed nuclei arised in spindle, enlarged or granular form with a weaker DAPI-labeling (Fig. 2h).

Discussion

The present study demonstrates, that cholinergic and dopaminergic neurons undergo cell death when incubated with rotenone, an inhibitor of complex I of the ETC.

Cholinergic and Dopaminergic Neurons in Organotypic Brain Co-Slices

Inside the organotypic brain slice model the basic cellular and connective organization of the donor brain regions are well preserved, thus the slice culture is able to maintain the survival of different cell types, the cytoarchitecture of the tissue, the connections between cells and neuronal properties. Therefore, the slice cultures provide an easily accessible experimental model for studies of toxic, degenerative and plastic regenerative or developmental changes in the brain [28]. The organotypic brain slice model resembles more closely the *in vivo* condition of a high density cell system, which separates it from dissociated nerve cell cultures. In slices, the individual cells are arranged in close contact and do not lose density-dependent regulatory mechanisms, three-dimensional architecture as well as tissue-specific transport and diffusion probabilities [29]. Gähwiler and colleagues [30,31] introduced the organotypic brain slice model as roller tube cultures. This technique was modified [32, 33], is meanwhile used by several research groups [34–37] and is well established in our research group [23, 24, 27, 38]. Moreover, the addition of growth factors into the culture medium prevents the neurons from cell death, which has been shown in previous studies by us [23, 24, 27]. The addition of growth factors is necessary for certain neuronal systems because the slices do not produce enough endogenous growth factors to support the survival of the neurons. In fact, we have previously shown that slices contain less than 3 pg NGF/mg tissue,

which is insufficient to support the cholinergic phenotype [23]. Similarly, GDNF is the most potent trophic factor for dopaminergic neurons and supports the survival of dopaminergic neurons in organotypic brain slices, as shown in previous studies in detail [27]. Additionally, co-cultures combined of two brain regions have been already studied and provide an excellent system to examine the interaction between two related brain areas, e.g. the combination of the nBM together with the cortex [24] or the vMes together with the dStr [27]. In the present study, we used a co-culture system composed of four brain regions, the nBM, vMes, dStr and cortex. The slices for these co-cultures derive from early postnatal day 7–9 rat brains and neurons at this developmental stage are very resistant for explantation, the cytoarchitecture is already established and the tissue has the property to flatten. The reason for this flattening to about 100–200 μm is completely unknown, but it is an internal mean for a good preparation and dissection. The organotypic brain slice model allows to study survival and neurodegeneration of these cholinergic neurons of the nBM as reported in several recent papers by us [23, 24, 39, 40]. Cholinergic neurons are labeled by the key enzyme ChAT and the number of ChAT-positive neurons strongly correlate with the survival rate. We have previously shown, that cholinergic neurons survive in co-slices of the nBM and cortex and cholinergic nerve fibers grew into the cortex slice [24]. Dopaminergic neurons are located in the vMes, consisting of the substantia nigra pars compacta and the ventral tegmental area [41, 42]. We have previously shown that dopaminergic neurons survive in single vMes slices [38] and in co-slices of the vMes and dStr [27]. Similarly to cholinergic neurons, dopaminergic neurons grew into the functional target, the dStr. Again, these nerve fibers can be regarded as new innervation from the ventral mesencephalon, because all nerve fibers in the axotomized dorsal striatum degenerate. Similarly to ChAT, we have used the key enzyme TH to label dopaminergic neurons, which is well established [27]. In summary, our co-slice model allows to study mechanisms of cholinergic and dopaminergic neuronal cell death in a well established interconnected in vitro model.

Effects of Rotenone on Cholinergic and Dopaminergic Neurons

Rotenone is an odorless chemical, broad-spectrum insecticide, piscicide and pesticide and inhibits with high affinity the complex I of the electron transport chain [18–22, 43]. This leads to the generation of reactive oxygen species (ROS) [19] resulting in neurodegeneration and apoptosis [21]. Rotenone is extremely lipophilic and thus it easily and rapidly crosses the blood-brain barrier and cell membranes without specific, active transporters [18, 19]. Rotenone has been used to simulate the pathological features of PD in rats [21, 22]. However, results of in vivo rotenone application were not uniform and loss of dopaminergic neurons in the vMes was not always achieved [19,20, 22]. Testing different doses of rotenone in our brain slice model showed, that only 10 μM rotenone but not lower doses exhibited cell death of cholinergic neurons in nBM slices. Therefore, we treated our co-cultures with 10 μM rotenone for 24 h, which is similar to the study of Keeney et al. [44]. While other studies also reported effects with lower doses of rotenone, those studies mostly tested primary cell cultures, which are likely more sensitive [43,45]. The organotypic brain slice model represents a stable three-dimensional system, where various cell types (e.g. astroglia) cooperate and work together to eliminate mild injured stimuli. This leads to the requirement of higher doses of rotenone to induce a detectable effect on the number of neurons in our co-culture system, compared to e.g. primary cell cultures. Again this

resembles more likely the in vivo situation, where higher doses are needed, because of metabolism and diffusion properties of the drug.

Only little is known about the effect of rotenone on cholinergic neurons. In the present study we have shown, that cholinergic as well as dopaminergic neurons degenerate after rotenone treatment. The remaining cholinergic and dopaminergic neurons appeared in smaller shape exhibiting apoptotic morphology. Previous studies support the hypothesis, that dopaminergic neurons in the vMes have an intrinsic sensitivity to complex I defects [19, 20,43]. Our data indicate, that also cholinergic nBM but not dStr neurons possess a higher susceptibility to complex I inhibition. Moreover, treatment of co-cultures with rotenone revealed several DAPI-positive malformed nuclei, which are clear signs of apoptosis. The increased TUNEL-positive staining [46, 47] in the nBM and dStr, as well as in the vMes indicates that apoptotic cell death occurred after rotenone application. The mechanism, which leads to cell death after rotenone treatment are not fully known, but it has been assumed, that application of rotenone conducts to an early rise in intracellular sodium, which seems to play a primary role in the rotenone response, independent of the neuronal system [48]. Taken together, rotenone induced cell death of cholinergic and dopaminergic neurons in a well established organotypic cell culture model. Our data show that the number of DAPI- or TUNEL-positive cells was much higher as the number of cholinergic or dopaminergic neurons. Thus, we suggest that the apoptotic cell death is not related to cholinergic or dopaminergic neurons, but to non-neuronal cells, such as e.g. astroglia. In fact we have recently shown that dopaminergic neurons do not exhibit apoptotic features after 6-OHDA lesions [27].

In summary, our data indicate that dopaminergic as well as cholinergic neurons degenerate after complex I inhibition. Further experiments are necessary to clarify, if inhibition of complex I of the ETC may play a role in initiation of early Alzheimer's disease.

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Fig. 1.

Co-slices composed of four brain regions, the basal nucleus of Meynert (nBM), dorsal striatum (dStr), ventral mesencephalon (vMes) and cortex (Ctx) were positioned on a 3 mm 0.4 μ m membrane insert (**a**), incubated for 4 weeks with nerve growth factor and glial cell line-derived neurotrophic factor and stained with cresyl violet (**b**). The different brain areas are indicated by *dashed lines*. Co-cultures displayed a positive staining for choline acetyltransferase-(ChAT) in the nBM and dStr (**c**, **d**), whereas, the cortex and vMes exhibited no ChAT-positive immunoreactivity (**e**, **f**). Dopaminergic neurons in the vMes were stained for tyrosine hydroxylase (TH) (**g**) and dopamine D2 receptor- (D2R) positive cells were found in the dStr (**h**). Scale bar in **b** = 620 μ m (**b**), 90 μ m (**b–h**)

Fig. 2.

Co-cultures after rotenone treatment. Four-week old co-slices composed of four brain regions were treated overnight without (**a, c, e, g**) or with 10 μ M rotenone (**b, d, f, h**) and were immunohistochemically stained against choline acetyltransferase (ChAT; **a-d**), or DAPI (**e, f**) or TUNEL (**g, h**). Note enhanced cholinergic cell death after rotenone treatment (**a-d**). Figure **f** represents enhanced TUNEL-positive nuclei after rotenone treatment. DAPI-positive malformed nuclei (spindle form, enlarged or granular) after rotenone treatment exhibited an apoptotic morphology (*arrow, h*) compared to well-formed, round and healthy nuclei (*arrow, g*). Scale bar in **a** = 250 μ m (**a, b**), 120 μ m (**c, d**), 90 μ m (**e, f**), 60 μ m (**g, h**)

Table 1

Dose-dependent effect of rotenone on cholinergic neurons in the basal nucleus of Meynert

Treatment	ChAT+-neurons	<i>n</i>	<i>P</i>
–	123 ± 10	15	–
10 µM	85 ± 7	14	**
1 µM	106 ± 7	15	NS
100 nM	110 ± 16	8	NS
10 nM	124 ± 27	6	NS
1 nM	113 ± 21	8	NS

Two week old brain slices of the basal nucleus of Meynert (nBM) were incubated with 10 µM, 1 µM, 100 nM, 10 nM or 1 nM rotenone or without (–) rotenone for 24 h and then for further 3 days without rotenone. Slices were then fixed, stained for choline acetyltransferase (ChAT) and the number of ChAT-positive neurons was counted in the brain slices. Values are given as mean ± SEM; *n* gives the number of analyzed slices. Statistical analysis was performed by one way ANOVA with a Fisher PLSD posthoc test

NS not significant

**
P < 0.01;

Table 2

Effects of rotenone on neuronal cell death in co-cultures composed of four brain regions

Region		Control	Rotenone
nBM	ChAT+	124 ± 15	77 ± 15**
	DAPI	9 ± 1	26 ± 3***
	TUNEL+	45 ± 5	238 ± 73**
vMes	TH+	81 ± 18	40 ± 9**
	DAPI	12 ± 2	51 ± 7***
	TUNEL+	53 ± 7	326 ± 63***
dStr	ChAT+	54 ± 12	32 ± 7 NS
	DAPI	8 ± 1	13 ± 1**
	TUNEL+	52 ± 3	153 ± 18*

Four week old co-culture brain slices ($n = 7$), composed of the basal nucleus of Meynert (nBM), the dorsal striatum (dStr), the cortex and the ventral mesencephalon (vMes), were incubated with 10 μ M rotenone for 24 h and then for further 3 days without. Slices were then fixed, stained for choline acetyltransferase (ChAT+) or tyrosine hydroxylase (TH+) or DAPI or DNA fragments were in situ labeled using the TUNEL assay. The number of ChAT+/TH+-positive neurons or DAPI/TUNEL+ cell was counted in the particular brain region. Values are given as mean $\pm$ SEM per ($260 \times 190 \mu$ m). Statistical analysis was performed by one way ANOVA with a Fisher PLSD posthoc test

NS not significant

 $P < 0.001$;

**
 $P < 0.01$;

*
 $P < 0.05$;