Improvement of mitochondrial function and dynamics by the metabolic enhancer piracetam

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Abstract
The metabolic enhancer piracetam is used in many countries to treat cognitive impairment in aging, brain injuries, as well as dementia such as AD (Alzheimer’s disease). As a specific feature of piracetam, beneficial effects are usually associated with mitochondrial dysfunction. In previous studies we were able to show that piracetam enhanced ATP production, mitochondrial membrane potential as well as neurite outgrowth in cell and animal models for aging and AD. To investigate further the effects of piracetam on mitochondrial function, especially mitochondrial fission and fusion events, we decided to assess mitochondrial morphology. Human neuroblastoma cells were treated with the drug under normal conditions and under conditions imitating aging and the occurrence of ROS (reactive oxygen species) as well as in stably transfected cells with the human wild-type APP (amyloid precursor protein) gene. This AD model is characterized by expressing only 2-fold more human Aβ (amyloid β-peptide) compared with control cells and therefore representing very early stages of AD when Aβ levels gradually increase over decades. Interestingly, these cells exhibit an impaired mitochondrial function and morphology under baseline conditions. Piracetam is able to restore this impairment and shifts mitochondrial morphology back to elongated forms, whereas there is no effect in control cells. After addition of a complex I inhibitor, mitochondrial morphology is distinctly shifted to punctate forms in both cell lines. Under these conditions piracetam is able to ameliorate morphology in cells suffering from the mild Aβ load, as well as mitochondrial dynamics in control cells.

Introduction
To fulfil the complex demands of providing the neuron with ATP, not only at the level of the cell body, but also at the synapses, mitochondria are dynamically regulated with the ability to undergo constant fission and fusion and to migrate from cell body to synapse and vice versa, the latter being quite important considering the substantial lengths of some axons [1,2]. Accordingly, mitochondria vary in localization, shape, size and number. In response to environmental stimuli, mitochondria fuse and divide constantly, to meet changes in cellular metabolic needs and to distribute the chondriome among the cell as a major repair mechanism [1,2]. Impaired mitochondrial dynamics induce synaptic dysfunction and finally the loss of synapses [1,2]. Substantial evidence suggests that all aspects of mitochondrial dynamics are relevant, not only to guarantee having sufficient mitochondria within the neurons at places of energy demand, but also as major mechanisms of mitochondrial repair and renewal.

Mitochondrial dysfunction in aging and AD (Alzheimer’s disease)
Distinct disturbances in mitochondrial functions occur in the aging brain whereof impairment of complex I of the respiratory chain is one of the most prominent [3,4]. An impaired respiratory machinery finally leads to elevated levels of ROS (reactive oxygen species). Elevated ROS, when it exceeds the cellular detoxifying capacity, finally results in accumulation of damaged proteins, altered mitochondrial membrane properties and lesions in mitochondrial nucleic acids (mtDNA). In AD, complex I and especially complex IV functions are additionally impaired [3–6], already in very early stages of the disease, most likely by the intracellular accumulation of Aβ (amyloid β-peptide) oligomers, as those changes are seen long before typical Aβ plaques can be detected. As major consequences, enhanced depletion of mitochondria in axons and dendrites, synaptic dysfunction, loss of synapses and neurites, and finally neuronal loss occurs, as typical early histopathological alteration not only of the AD brain but also of the brain during aging. Moreover, elevated ROS production by impaired complex I and complex III function elevates Aβ production by stimulating γ-secretase activity [6]. A vicious cycle is generated which leads to severe impairment of cognitive function, enhanced apoptosis and finally massive loss of brain volume.

Key words: aging, Alzheimer’s disease, mitochondrial morphology, piracetam.

Abbreviations used: Aβ, amyloid β-peptide; AD, Alzheimer’s disease; APP, amyloid precursor protein; APPsw, Swedish APP mutation; HEK, human embryonic kidney; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; SOD, superoxide dismutase.

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In line with the large body of evidence indicating mitochondrial dysfunction as a major pathomechanism in AD [7,8], several reports indicate deficits of mitochondrial dynamics in this disease, including impaired balance between fission and fusion mechanisms and reduced mitochondrial trafficking [9,10]. This dysbalance results in a pronounced fragmentation of mitochondria, which is a correlate for the enhanced fission processes taking place. Moreover, interfering with impaired mitochondrial dynamics has been proposed as a novel strategy for anti-dementia drugs [11–13].

**Piracetam: a metabolic enhancer**

Piracetam, the prototype of the so-called ‘nootropic’ drugs [14] is used in many countries to treat cognitive impairment in aging and brain injuries, as well as dementia [15]. As a specific feature of piracetam and other similar compounds [15–17], beneficial effects on cognition are usually associated with impaired brain function such as aging, hypoxia, glucose deprivation, injuries or even neurodegeneration. As all of the pathological conditions mentioned above are typically associated with the vicious cycle between enhanced oxidative stress, elevated ROS production, mitochondrial damage, reduced energy supply and enhanced ROS, we speculated that piracetam might enhance mitochondrial function or at least protect against mitochondrial damage under the aforementioned conditions, but mainly in brain aging or dementia.

**Improved mitochondrial function in brain aging and AD by piracetam**

The first evidence that piracetam might enhance mitochondrial function originates from an older observation that piracetam improved glucose uptake and utilization in animal and human brains and also led to elevated ATP levels [15]. On the basis of those findings, the term metabolic enhancer has been used to characterize piracetam’s beneficial properties in men [15]. Accordingly, we speculated that piracetam might enhance mitochondrial function or at least protect against mitochondrial damage in situations of enhanced damage.

To investigate this assumption, we used different mouse and cell models and induced experimentally oxidative stress using different approaches to mirror within hours or a few days what is usually seen in aged or diseased brains at the end of the lifespan. Mitochondrial function was assessed by monitoring MMP (mitochondrial membrane potential) using specific membrane dyes, by measuring ATP production and the release of pro-apoptotic factors [18,19]. In agreement with this hypothesis, piracetam improved MMP and elevated ATP production in several cell models following impairment after ROS generation by H2O2 or sodium nitroprusside, serum deprivation or Aβ treatment (Table 1). Additionally we used transgenic mice overexpressing human Aβ to verify these *in vitro* situations *in vivo*. Beside improved mitochondrial function, measured via MMP and ATP levels, we observed reduced Aβ levels, which give rise to the suspicion that piracetam might interfere with the aforementioned vicious cycle by protecting mitochondria. As the pronounced loss of neurites and synapses in AD brain is one of the functionally most relevant histopathological lesions [4], we decided to investigate neurite outgrowth in PC12 cells. Treatment with oligomeric Aβ1–42 alone reduced neuritogenesis significantly, whereas piracetam is able to inhibit this negative effect completely. These observations were confirmed using PC12 cells transgenic for the human APPwt [wild-type APP (amyloid precursor protein)] and APPsw (Swedish APP mutation) gene, where again piracetam is able to reverse the impaired neurite outgrowth compared with control cells [19].

To study processes occurring in the aging brain, we assessed 22-month-old NMRI mice fed with 500 mg/kg piracetam. Beside the amelioration of impaired MMP, piracetam was able to decrease the activity of different antioxidative enzyme activities such as glutathione peroxidase, glutathione reductase and SOD (superoxide dismutase), which are normally elevated in aged brain compared with those of young animals. In agreement with earlier work, young mice benefit less from piracetam treatment, supporting the assumption that the effectiveness is always much more pronounced under impaired conditions (Table 1).

We have recently shown [6] that brain aging via enhanced oxidative stress and the typical histopathological alterations in AD synergistically impair mitochondrial function [8,16].

### Table 1 | Piracetam’s beneficial effects on different measures of mitochondrial function in cell culture and animal models of aging and AD

<table>
<thead>
<tr>
<th>Subject</th>
<th>Effect</th>
</tr>
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<tbody>
<tr>
<td>NMRI mice</td>
<td>SOD, GR and GPx↓ in aged animals</td>
</tr>
<tr>
<td>Tg AD mice</td>
<td>ATP production↑</td>
</tr>
<tr>
<td>HEK APPwt</td>
<td>MMP↑</td>
</tr>
<tr>
<td>PC12 cells</td>
<td>ATP production↑ after serum deprivation or SNP insult</td>
</tr>
<tr>
<td>PC12 APPwt/sw</td>
<td>Neurite outgrowth↑ after Aβ1–42 or SNP insult</td>
</tr>
</tbody>
</table>

Data are summarized from Keil et al. [18] and Kurz et al. [19]. HEK APPwt, HEK-293 cells stably transfected with human APPwt; PC12 APPwt/sw/HEK PC12 cells stably transfected with human APPwt or the Swedish APP mutation; Tg AD mice, C57BL/6 mice bearing the human Swedish and London mutations in the human APP. GPx, glutathione peroxidase; GR, glutathione reductase.
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Figure 1 | Rotenone and piracetam induce mitochondrial shape change in HEK-293 cells

(A) Rotenone treatment (25 μM) for 24 h shifts mitochondrial morphology significantly towards punctate forms. Data are expressed as means ± S.E.M. (n = 8). ***P < 0.001 compared with untreated control cells, two-way ANOVA with Bonferroni post-test. Data are modified from Kurz [25]. (B) Preincubation with piracetam for 6 h ameliorates rotenone-induced impairment of mitochondrial morphology in a concentration-dependent manner. Data are expressed as means ± S.E.M. (n = 8). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with rotenone control, two-way ANOVA with Bonferroni post-test (C. Stockburger, K. Leuner and W.E. Müller, unpublished work).

Table 2 | Mitochondrial morphology in SY5Y cells (percentage of mitochondria)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Punctate (&lt;2 μm)</th>
<th>Truncated (2–4 μm)</th>
<th>Tubular (4–10 μm)</th>
<th>Elongated (&gt;10 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SY5Y Mock</td>
<td>16.57 ± 3.11</td>
<td>34.00 ± 2.64</td>
<td>42.29 ± 3.75</td>
<td>7.14 ± 1.45</td>
</tr>
<tr>
<td>SY5Y Mock + Rot</td>
<td>42.57 ± 4.73***</td>
<td>39.57 ± 2.53</td>
<td>17.14 ± 2.87***</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>SY5Y Mock + Rot + Pira</td>
<td>28.71 ± 3.15***</td>
<td>42.14 ± 1.96</td>
<td>28.43 ± 2.64*</td>
<td>0.71 ± 0.42</td>
</tr>
<tr>
<td>SY5Y APP&lt;sub&gt;sw&lt;/sub&gt;</td>
<td>52.14 ± 2.45***</td>
<td>33.14 ± 2.18</td>
<td>14.00 ± 0.98***</td>
<td>0.714 ± 0.29</td>
</tr>
<tr>
<td>SY5Y APP&lt;sub&gt;sw&lt;/sub&gt; + Pira</td>
<td>35.43 ± 3.94***</td>
<td>39.71 ± 1.29</td>
<td>23.29 ± 3.14*</td>
<td>1.57 ± 0.48</td>
</tr>
</tbody>
</table>

Piracetam improves mitochondrial morphology

We have previously reported that HEK (human embryonic kidney)-293 cells stably transfected with human APP<sub>sw</sub> gene show mitochondrial deficits typical for other cell or animal models of early stages of AD [6,8,9,11], including substantial changes of mitochondrial morphology (tubular compared with fragmented shape) [6,13]. Using a sensitive laser-scanning microscopy technique, we were able to confirm and extend the pronounced alterations of mitochondrial morphology after complex I inhibition by rotenone for normal HEK-293 cells (HEK<sub>ut</sub>). In HEK<sub>ut</sub> cells, a predominance of tubular and truncated mitochondria show up, whereas in rotenone-treated cells the proportion of punctate mitochondria strongly increases (Figure 1). Piracetam at concentrations within the range used in our previous experiments had no leading again to further increases of ROS production. As Aβ production appears to be activated by oxidative stress via γ-secretase activation by ROS, Aβ will activate its own production and finally create a vicious cycle which finally leads to the pronounced alteration of AD brain. In agreement with this model, piracetam has been shown in our experiments to reduce Aβ production following ROS generation or elevated Aβ production [19]. The data presented clearly indicate that piracetam protects mitochondria against different conditions associated with oxidative stress including aging as well as Aβ toxicity and/or overload. The concentrations of piracetam effective in vitro (100–1000 μM) and the doses used in the in vivo experiments (100–500 mg/kg) are quite well within the plasma concentrations seen in patients treated with the standard dose of approximately 5 g daily, which range between 200 and 2000 μM [15].
effect on mitochondrial morphology in HEKut control cells under baseline conditions. However, cells substantially responded to piracetam treatment after an insult with rotenone in a concentration-dependent manner, where piracetam-treated cells show mitochondrial shape distribution similar to the HEKut control cells (Figure 1). To confirm our findings in a neuronal cell line, we used SY5Y cells, which responded similarly to complex I inhibition by rotenone with a large increase in punctate mitochondria and a substantial decrease in elongated mitochondria (Table 2) [20]. Again, piracetam treatment using the same concentration reversed this effect nearly back to normal (Table 2). We additionally investigated the effect of piracetam in SY5Y cells stably transfected with an additional human APP gene (APPwT). These cells have previously been shown to exhibit many features of mitochondrial dysfunction by an elevated Aβ production of approximately 2-fold [20]. Relative to vector control, elevated Aβ in SY5Y cells again leads to typical changes of mitochondrial dynamics, similarly to our observations in HEK-293 cells [6,13]. Piracetam treatment partially reversed these changes by shifting the balance from fission (punctate) to fusion (tubular and elongated) (Table 2). In summary, these data clearly indicate that the metabolic enhancer improves mitochondrial function following mitochondrial impairment typical for brain aging and early stages of AD. Although piracetam does not interact with any specific target in human brain (receptor, enzyme, transporter, ion channels etc.), it binds to the polar phospholipid headgroups of membranes and thereby improves the fluidity of neuronal as well as mitochondrial membranes [15,17,21,22], especially after situations leading to reduced membrane fluidity such as oxidative stress. Since the fluidity of mitochondrial membranes seems to be an important regulator not only of mitochondrial function but also of mitochondrial dynamics [22–24], it is reasonable to assume that piracetam’s effects on mitochondrial dynamics can be explained by its regulation of membrane fluidity.

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**References**