Abstract. This review supports the necessity of combining fundamental chemical and biological methods to scrutinize potential causative agents in neurodegeneration. This is supported by recent experimental evidence in relation to the use of nicotine as a potential therapeutic agent, especially when following the path of iron’s role in catalysing the generation of reactive oxygen species via a Fenton like reaction. Exploration of the dose-response relationship indicates that acute administration offers the most likely success, reducing tremor and improving cognitive performance amongst others. Confirmation of this relationship is gathered from recent in vivo and in vitro efforts that support this hypothesis.

As stated by Kienzl et al. (1) "the search for powerful modern methodologies has to proceed from a molecular level to the cellular level and from there to the whole CNS." The significance of this statement is evermore apparent with development in modern technologies. Similarly, as the boundaries between chemistry and biology are increasingly ill defined, the benefits of both a chemical and biological approach towards an understanding of complex biochemical reactions is inevitable. This can be seen in the increasing use of highly specialized chemical methods that simulate in vitro, as "proof of concept", various stages of known molecular pathways probing the kinetics and mechanisms that are of essence in the understanding of various biochemical reactions. Additionally there is an increase in the application of in vitro experimentation to examine the potential role of suggested therapeutics to inhibit, in a beneficial way, any molecular pathway that may contribute to the degeneration of neurons.

This review highlights the in vitro contributions that have assisted in the understanding of complex molecular pathways. Given the consistent controversy (2) over these epidemiological results regarding the bias of a study towards artefact and design, especially with a topic as sensitive as nicotine, it is accepted throughout this review and in agreement with many others that iron possesses a degenerative role in the onset of a broad range of diseases (see 3). The first part of this review highlights the chemical pathways of various known neurotoxins and their interplay with iron to suggest how neuro-degeneration may occur. Then the current in vivo evidence supporting the therapeutic role of nicotine is discussed along with the potential mechanisms that may be initiated, special relevance being paid to the interplay between iron and nicotine. Finally the current chemical understanding of iron and nicotine’s interactions is discussed.

Interestingly, in the past 10 years there have been more published results indicating a broad range of areas where nicotine possess therapeutic benefit as opposed to being of little or no benefit. This is contrary to a short report issued by Piggot et al. (4), at the beginning of the 1990’s, concerning smoking and Parkinson’s disease (PD), which highlighted the unexplored potential of nicotine receptors in the modulation of PD, hence instigating this report.

Molecular pathways related to neuronal cell death and suggested oxidising properties of iron via a Fenton reaction

A pathological hallmark of idiopathic PD is a progressive loss of neuromelanin-containing dopamine (DA) from the substantia nigra pars compacta (SNPc). It is generally accepted that one of the causative effects of this progressive loss of dopaminergic neurons is due to the increased generation of neurotoxins that somehow induce neuronal cell death. Amongst the varying number of fields and specialists studying neurodegeneration, there is a general consensus that neurodegeneration can be initiated either by

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genetic or environmental factors i.e., nature versus nurture. However, it is the biochemical pathways of the relevant neurotoxins that have been indicated to possess apoptotic properties regarding cell death that is discussed. Additionally, the predominance of iron as a catalyst for all these reactions is explained.

Neurotoxicity of 6-hydroxydopamine. 6-Hydroxydopamine (6-OHDA), illustrated in Figure 1, is one of the most common neurotoxins used in experimental models of neurodegeneration. Is it is a hydroxylated analogue of the natural neurotransmitter DA (Figure 2).

6-OHDA was originally isolated by Porter et al. in 1959 (5,6) and first suggested as possessing detrimental biological effects by Senoh et al. (7-10) Later, Stone et al. (11) demonstrated that 6-OHDA induces noradrenalin depletion in the autonomic nervous system of the heart. Subsequently, several studies demonstrated its ability to destroy nerve endings of sympathetic neurons (12-15). As 6-OHDA is unable to cross the blood-brain barrier, in vivo studies often involved systematic injection via intracerebral administration. Experimental models, mainly using rats, show that when 6-OHDA is injected into the SNpc nigral dopaminergic neurons are destroyed and the striatum of DA transmitters are depleted resulting in pathological symptoms similar to PD. Recent studies have suggested that 6-OHDA can be considered as a physiological endogenous neurotoxin. In fact, several studies have reported the presence of 6-OHDA in both rat (7-10) and human brain (16) as well as in urine of l-dopa-treated patients (17).

It is known from previous clinical studies (18) that nigral dopaminergic neurons contain significant levels of DA, hydrogen peroxide and free iron (19). Several mechanisms have been postulated for the generation of 6-OHDA:

Non-enzymatic reactions: In an in vitro study (20,21), the reactions of DA, 5-hydroxydopamine (5-OHDA) and 6-OHDA, with molecular oxygen - in the presence or absence of catalytic amounts of Fe(III) and other metal ions - were investigated and the implication of these results with respect to the chemistry involved in the progress of Parkinson's disease was highlighted. However, 6-OHDA reacts without showing the necessity for such an intermediate, and it was demonstrated as possessing the capability of reducing iron within ferritin, allowing it to be released as Fe^{2+}. This reaction was demonstrated to proceed via an outer sphere electron transfer reaction, in a manner similar to the mitochondrial electron transport chain.

Oxidative stress and neurodegeneration: Another theory that has been receiving increasing attention with respect to the role of 6-OHDA in PD is that of oxidative stress. In 1971 Heikkila and Cohen (22) suggested that 6-OHDA was capable of inducing nigrostriatal dopaminergic lesions via generation of hydrogen peroxide and/or derived hydroxyl radicals, collectively known as reactive oxygen species (ROS). Several in vivo (23-25) and in vitro (26-29) studies confirmed that this oxidative stress could indeed result in direct damage to mtDNA. The generation of ROS may arise from two distinct mechanisms, namely deamination by monoamine oxidase (MAO), specifically located in the mitochondria and auto-oxidation, as mentioned above. Deamination results in ROS since 6-OHDA is a substrate for MAO (30,31), resulting in the generation of ROS as a side product of this enzymatic reaction.

Whilst, the generation of ROS seems more likely to be a result of initial non-enzymatic auto-oxidation, there is a possibility that the generation of ROS from MAO may be amplified in the presence of free iron. This has been demonstrated by the increased levels of iron in the SNpc and striatum subsequent to injections of 6-OHDA (32-34). The role of iron in the toxicity of 6-OHDA is also suggested in studies that show direct injection of iron into the SNpc (35,36) produces similar effects. Furthermore, these 6-OHDA induced deleterious effects were prevented by iron chelating agents (37,38).

Neurotoxicity of dopamine. Dopamine, Figure 2, is a natural neurotransmitter that in normal circumstances is mainly contained in vesicles which modulate the concentration of DA both in the cytoplasm and in the synaptic cleft.
certain pathological conditions, such as ischemia or hypoxia, increases in extra or intracellular levels of DA have been suggested to lead to brain damage (39). Additionally, experiments conducted both in vivo (40) and in vitro (41-43) indicate that DA is toxic in a variety of neuronal and non-neuronal cells. Additionally DA reacts as a precursor to the above-mentioned reactions for 6-OHDA.

**Non-enzymatic reaction of dopamine:** In the presence of O2, DA was shown (20,21) to react spontaneously without the necessity of metal-ion catalysis in the presence of stoichiometric amounts of H2O2, to form initially pink oxidised melanin. DA was also demonstrated to react with Fe(III) yielding an intermediate 1:1 complex, which decomposes releasing Fe(II) and the semiquinone, that can react further with iron and other enzymatic systems generating more ROS (44). Also, it is shown (in vitro) that Fe(II) reacts in a Fenton type reaction, \( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \bullet \text{OH} + \text{OH}^- + \text{Fe}^{3+} \) (1)

These results supported the non-enzymatic reactions postulated by others (45): for example, it was shown (46) that melanin could increase free iron levels by displacing it from ferritin, hence amplifying the chemical reaction between DA and iron. Additionally, in the presence of nitrite ions, DA can be oxidised by a spectrum of hydrogen peroxide dependent systems giving small quantities of 6-OHDA and 6-nitrodopamine.

**Oxidative stress of dopamine:** Similarly to 6-OHDA, DA is a substrate for MAO (47,48) which gives rise to hydrogen peroxide via this enzymatic reaction. The relevance of this is explained in the section on oxidative stress and neurodegeneration.

**Neurotoxicity of MPTP.** Interest in the role of MPTP, (Figure 3) was instigated in the late 1970's when a group of young people, addicted to a new synthetic drug that was a derivative of fentanyl, developed symptoms very similar to PD (49) such as bradykinesia, rigidity, postural instability and resting tremors (50).

Analysis of this drug revealed that it contained at least 3% MPTP (51). As a consequence of these findings MPTP was considered to be a powerful drug capable of inducing nigral degeneration and hence is being considered here (for a complete review readers are referred to Tipton et al. (52) and Gerlach et al. (53)). It was, however, recognised that older animals were more susceptible than younger ones to MPTP (54) in a similar manner that other known toxins such as 3-nitropropionic acid are age-dependent (55).

**Non-enzymatic reaction of MPTP:** Similar to 6-OHDA and DA, increased iron may be of great importance in MPTP toxicity triggering a Fenton-like reaction in dopaminergic cells. Studies show that, in the presence of MPTP, there is an increase in iron levels within the SNpc (56,57). Further studies have supported this role of free iron, as chelators such as desferrioxamine, with known specificity for Fe(III), prevented progression of cell degeneration (58).

**Oxidative stress of MPTP:** Unlike 6-OHDA, MPTP is able to cross the blood-brain barrier, mainly glial cells, where it is converted to its effective form 1-methyl-4-phenypyridinium (MPP+) in the presence of MAO B –explaining the role of MAO-I B inhibitors against MPTP neurotoxicity. Free cytoplasmic MPP+ enters the mitochondria via an energy-dependent (59) mechanism inhibiting the activity of this organelle, resulting in a drop in cellular ATP levels, alteration in membrane potential and subsequently cell death. There are several lines of evidence suggesting the role of ROS in MPT P-induced neurotoxicity.

**The potential role of iron in cellular processes.** Iron (Fe) is a transition element as its ions having partially filled orbitals, occur in two principle oxidation states, ferrous Fe2+ (d6) and ferric Fe3+ (d5). The binding strength of Fe2+ to ligands and the intermediate concentration of free Fe ions within the human brain suggest that it may play a role in catalytic and regulatory processes in the cell (3). Transition metals are capable of accepting or donating single electrons to promote redox reactions and free radical formation. Additionally, and as outlined above, the most commonly occurring factor in inducing the onset of neurotoxin production appears to be associated with the presence of free iron. Further support of the role of iron in neurodegeneration is that the human brain Fe content is highest in the SNpc, globus pallidus, red nucleus and putamen (60) – all areas linked with neuronal cell death in degenerative diseases. Within the central nervous system (CNS), most bound iron is stored in an inactive form bound to intracellular ferritin, until some event initiates the onset of cell death. It is for this reason that
many current research efforts regarding therapeutic methods for neurodegeneration are focussing on methods of eliminating or reducing iron overload within the body, utilising the very bond strengths that make it a destructive catalyst for neurotoxin production.

The biological effects of nicotine
Nicotine is a weak base with a pKa of approximately 8.0. At physiological pH, approximately 30% is non-ionised and can readily cross cell membranes. It takes approximately 10-20 seconds for nicotine inhaled from cigarette smoke to pass through the brain. The majority of nicotine inhaled via cigarette smoke is metabolised in the liver, although some is metabolised in the lungs. However, nicotine readily crosses the blood-brain barrier and is distributed throughout the brain (61,62). Its uptake into the brain appears to occur via passive diffusion and active transport by the choroids plexus (63). Approximately 70-80% of nicotine is metabolised to cotinine and the rest to nicotine-N'-oxide, (Figure 4).

Cotinine then undergoes a further metabolism to 3'-hydroxycotinine, the most abundant metabolite found in urine samples of smokers (Figure 4) (64-66). Within the brain, nicotine specifically binds to nicotinic cholinergic receptors, possibly located on pre-synaptic sites, an activation of several central nervous systems, neuro-hormonal pathways occurs (67,68) leading to the release of acetylcholine, DA, norepinephrine, serotonin, vasopressin, growth hormone and adrenocorticotropic hormone (69-71). It is within this context that the intracellular role of nicotine may provide therapeutic benefits via stimulation of the various neurochemical pathways. One aspect of nicotine’s therapeutic effect that will not be considered here is its role in inhibiting MAO enzymatic generation of ROS as any oxidative reactions are secondary processes and the scope of this review is focused on the anti-oxidant properties of nicotine.

Nicotine and neurodegeneration: In vivo evidence supporting the therapeutic benefits of nicotine. Over the past 40 years several epidemiological studies have demonstrated an inverse dose-response relationship between smoking and PD (72-75). One report (76) demonstrated that, when nicotine was administered transdermally, improvements related to attention and information processing were observed for Down’s syndrome patients compared with healthy controls. Another example involving a patient with long-standing spastic dystonia noted an improvement in symptoms after smoking cigarettes (77). Similarly, there is increasing evidence supporting nicotine’s therapeutic role in Tourettes Syndrome as studies have found that a chronic administration of nicotine reduced DOI (1-2,5-dimethoxy-4-iodophenyl-2-aminopropane) – induced head twitches (78). Such a hypothesis can be cross-examined by using various high standard analytical techniques to probe the consequence of nicotine, administered transdermally or otherwise. The use of paralell experimental methods and retrieval of similar experimental trends/results always strengthens the argument for fact. For example, to evaluate the efficacy of chronic nicotine administration in dementia, Hirata et al. (79) studied the event-related potentials (ERP) and the midlatency responses (MLR). This study was conducted on 22 individuals, 16 of whom suffered from some form of dementia (vascular dementia, Alzheimer’s disease and PD). Administration of nicotine was achieved transdermally from a nicotine patch (22.50-55 mg/day) for a period ranging from 2-4 weeks. The conclusion reached by this team of researchers was that chronic administration of nicotine might be beneficial in dementia as a cognitive enhancer.

Potential mechanism: As molecular reactions do not exist in confined spaces, there are a multitude of explanations for where nicotine’s therapeutic benefits may be observed. For
example, one study (80) postulated that nicotine activated
the nigrostriatal dopaminergic pathway and increased the
release of DA in the striatum. Piggot et al. (5) also
suggested that modulation of DA D2 receptors by cigarette
smoke could be a plausible explanation for the protective
influence of smoking. To elucidate the neuroprotective
effect of nicotine and to investigate its ability to attenuate
neurotoxic effects associated with Alzheimer's disease,
Semba et al. (81) conducted a study using rat hippocampal
culture. Exposure of these cultures to dexamethasone and
subsequently to nicotine resulted in a neuroprotective effect
that was attributed to: 1) nicotine antagonizing a
glucocorticoid-induced decrease of glucose uptake; 2)
induction by nicotine of certain types of neurotrophic
factors, or 3) regulation by nicotine of genes that may
interfere with the normal function of the hippocampal
region of the brain. Janson et al. (82) implemented a variety
of neuroanatomical techniques to investigate the
consequences of chronic continuous nicotine treatment on
the lesion-induced effects of a partial meso-diencephalic
hemitranssection. Both the striatonigral substance P (SP)
and the nigrostriatal DA pathways were studied. The results
of this study indicated that the lesion-induced degenerative
change was most pronounced in the lateral parts of the
ipsilateral substantia nigra and striatum. The main finding
of this study is that nicotine induced a disappearance of SP
immunoreactive nerve terminals in SNPc on the lesioned
side. These data were interpreted on the basis of previous
electrophysiological findings, where nicotine under similar
experimental conditions counteracted the lesion-induced
increase in firing in vivo in nigral DA neurons. Taken
together, these results indicate that nicotine may act by
reducing the SP excitatory input to the nigral DA cell, thus
rescuing them from dying. It is likely that the surviving cells
are functional, since increased extracellular striatal DA
levels were observed after nicotine treatment ipsilateral to
the lesion in a previous microdialysis experiment in vivo.

Similarly, findings by our group (21) demonstrated that
adult male rats when treated with nicotine showed a greater
memory retention than control studies in a water maze test,
however a neurochemical analysis of the neocortex,
hippocampus and the neostriatum of these same animals revealed that nicotine treatment had no effect on the
formation of ROS or lipid peroxidation for any of the brain
regions studied. Here it was suggested that the beneficial
effects of nicotine might be, at least partially, due to anti-
oxidant mechanisms, related to the presence of free iron.
Quik et al. (83) evaluated the neuroprotective effects of
nicotine against nigral neuronal damage by using
mesencephalic neurons in culture and treated them with a
selective dopaminergic neurotoxin. Their results indicated
that pre-treatment with nicotine protected against
dopaminergic nigral neural degeneration, again supporting
the theory that the nicotinic receptor ligands may be useful
anti-oxidants in PD therapy.

A autoradiographic study (84) of striatal nicotine
showed an increase in the release of neurotransmitters,
including DA, in patients suffering from PD and
Alzheimer's disease. This study was instigated due to
findings (85) indicating that far more needed to be
discovered concerning the contribution of receptor subtype
t.e., subunit abnormalities of neurons, to clinical symptoms
of neurodegeneration. Typical clinical symptoms studies
include changes in attention and cognition in general, mood
or levels of anxiety, pain perception or even the process of
conscious awareness. Again support of nicotine as a
therapeutic agent was reported.

While the above-mentioned mechanisms are not
extensive regarding the potential biochemical pathways that
may be involved in the therapeutic benefits of nicotine, they
do highlight those pathways where increase in iron content
may be playing a catalytic role.

Nicotine and neurodegeneration: In vitro evidence supporting
the therapeutic benefits of nicotine. As stated earlier (20,21),
DA oxidised by either the MAO or the auto-oxidation route
generates H2O2. Iron was shown to catalyse DA auto-
oxidation and the Fenton reaction to produce OH radicals
(86). In vitro studies carried out by Kienzl et al. (1)
investigated the ability of iron to catalytically influence the
generation of many of the neurotoxins mentioned above. In
vitro studies carried out in our laboratory using sopped-flow
spectrometry demonstrated: 1) that the presence of Fe²⁺
and $\text{H}_2\text{O}_2$ efficiently converted DA into the cytotoxic 6-OHDA via a Fenton-type reaction. pH titrations demonstrated that the oxidation of 6-OHDA by $\text{Fe}^{3+}$ proceeded without the prior formation of a metal ligand complex, as this reaction was capable of occurring via an outer sphere electron transfer between the oxygen of 6-OHDA and the d orbital of $\text{Fe}^{3+}$ ion.

A common method employed to study the generation of ROS is high performance liquid chromatography (HPLC). This approach is based on the quantification of hydroxylation products as a consequence of the reaction of OH radicals with both phenol and salicylic acid (87). Aromatic compounds are known to react with OH radicals, at fast rates (nanoseconds) forming a specific set of hydroxylated products, most often salicylic acid (88), that are easily analysed with HPLC (89,90). A HPLC study (91) that was also conducted in our laboratories analysed the effect of nicotine on products of this Fenton reaction, illustrated in Figure 5.

Here it was found that while low concentrations ($\leq$100 $\mu$M) had little or no effect on Fenton activity, at high concentrations (100-500 $\mu$M) of nicotine there was a significant decrease in Fenton activity (91). A similar HPLC study (92) was conducted examining the anti-oxidant properties of nicotine on the action of MPP$^+$ in the presence of iron. Again a reduction in the products of Fenton activity was noted. Another HPLC study (93) examined the effect of nicotine on striatum levels of MPP$^+$. Again, a dose-dependent relationship was observed. To find out whether a complexion reaction occurs between Fe(II)/(III), an electrochemical investigation was also carried out (94). It was observed that higher concentrations of nicotine resulted in a shift of the electrochemical behaviour from reversible to quasi-reversible, indicating the formation of some complexion reaction occurring. Titrations investigating the pH dependence of nicotine and its ability to form a complex with iron were also carried out. It was indicated that nicotine did indeed appear to form some weak complex, albeit not chelate, with Fe, but its formation was greatly dependent on pH, agreeing with others (95).

**Conclusion**

As described above, the number of molecular pathways that may be affected by nicotine are numerous. The general conclusions that have been reached to date by many researchers is that nicotine increases the amounts of DA within the central nervous system – mimicking the currently prescribed l-dopa-treatment, and alleviating symptoms associated with neurodegeneration. A second mechanism beginning to be explored involving nicotine’s therapeutic role is its ability to complex free iron, as this metal is highly associated with the progression of PD and other neurodegenerative diseases.

To date the results are not conclusive enough to support the role of nicotine as the only therapeutic agent against neurodegeneration and as this review highlights the need for consistency in experimental design. Supporting this statement is the concurrence of *in vitro* experiments with the effects noticed when studying the dose-response relationship *in vivo* (81); *i.e.* acute administration producing the greatest improvement in symptoms of PD (79-85). Our recent *in vitro* attempts indicate that high concentrations of nicotine have a chelating effect when compared with lower concentrations (91,94).

The chemical evidence of these results may offer an explanation for conflicting opinions with respect to the success of nicotine experiments conducted *in vivo*; *i.e.*, the dosages used were not "strong" enough to elicit a therapeutic benefit (93). In some instances (91,94) *in vivo* reports attribute the protective affect of nicotine to its ability to act as a ligand specific for $\text{Fe}^{2+}$. To date, *in vitro* results would indicate that there is weak formation of complexes between nicotine and $\text{Fe}^{2+}$ that may contribute to its neuroprotective effect (91). The chemical investigations carried out are, however, not exhaustive and further research is necessary. For instance, further chemical investigation into the reactions of metabolic intermediates with iron are needed, as it may be possible that hydroxylated nicotine could react via an outer sphere electron transfer reaction in a manner similar to 6-OHDA, which would result in removal of iron and further catalyse rather than inhibit any reaction of iron (97). Indeed the reverse situation may also be true; the metabolites of nicotine may have more enhanced chelating abilities than nicotine itself.

The current status of research appears to have a more fundamental approach towards the understanding of many neurological problems. However, more consistency in experimental design is needed to ensure that a definite result may be reached regarding many new therapeutic agents.

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