# Chapter 6. Evaluation of the neuroprotection of the reported selective neuronal nitric oxide synthase inhibitor 7-nitroindazole using the MPTP model of neurotoxicity

## 6.1. Research rationale and background

The possible role of nNOS and NO in cytotoxicity has caught the attention of many research groups.[227,228,256] The presence of excess NO has been associated with cell death in many neurological diseases (Chapter 2, section 2.2). Research groups are looking at the neuroprotective characteristics of inhibitors of nNOS with several type of inhibitors (section 2.2). Recently, it was reported that the inhibition of nNOS by 7-nitroindazole (7-NI, **113**, section 2.4) leads to protection against MPTP and methamphetamine (METH) neurotoxicity in treated mice and baboons.[227,229,257,258] 7-NI is one of the newer inhibitors and is described in the literature as a relatively selective nNOS inhibitor in vivo.[219,220,222,223] The neuroprotective effects of 7-NI were reported not to be related to inhibition of the MAO-B dependent conversion of MPTP to $MPP^+$ since 7-NI had no effect on the MAO-B catalyzed oxidation of benzylamine by mouse brain mitochondrial preparations.[227,228,259] Furthermore, the striatal levels of $MPP^+$ after MPTP treatment were reported to be the same with or without pretreatment with 7-NI.[227]

Consideration of the structural features of 7-NI, however, indicate that 7-NI is likely to interact with MAO-B since a wide variety of planar heterocyclic systems have been shown to possess MAO-B inhibition properties.[230-233] Another indication that 7-NI possibly interacts with MAO-B is the observation

that 7-NI treatment only results in elevated striatal dopamine levels.[227] A similar effect can be observed with other MAO inhibitors such as pargyline and (R)-deprenyl and is propably due to decreased DA metabolism. Therefore the possible inhibitory effect of 7-NI on the MAO-B mediated conversion of MPTP needs to be examined in greater detail. In vitro results obtained in our group showed that 7-NI indeed is a competitive inhibitor ($K_i$ = 40 μM)[234] and has led us to examine the effect of subcutaneous (s.c.) 7-NI pretreatment on striatal $MPP^+$ levels in C57Bl/6 mice treated intraperitoneally (i.p.) with MPTP. These in vivo studies employed a robust liquid chromatographic-diode array (LC-DA) assay that provides quantitative estimations of MPTP, $MPDP^+$, $MPP^+$ and 7-NI. Differences in the retention times and chromophores made it possible to monitor each compound on a different channel without overlapping peaks or interference from biological background. Figure 17 shows the chromatogram after injection of a solution containing all four analytes.

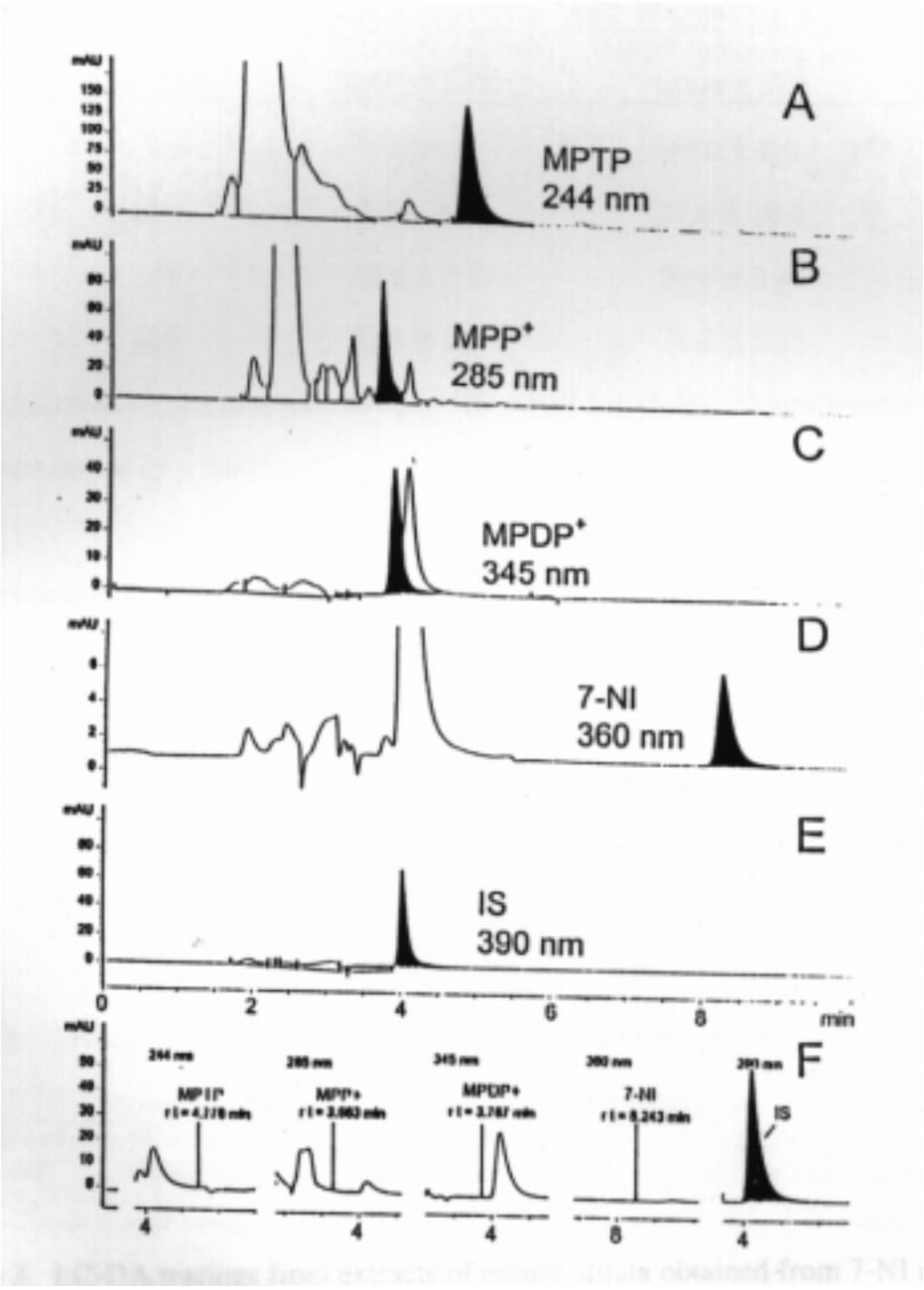
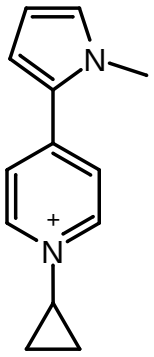



**Figure 17.** LC-DA Tracings from Extracts of Mouse Striata Obtained from 7-NI and MPTP Treated Animals (Panels A-F).

Identification of panels: MPTP (panel A), $MPP^+$ (panel B), $MPDP^+$ (panel C) and 7-NI (panel D). IS (panel E) was added to each of the isolated striata prior to analysis. Panel F is a composite tracing corresponding to panels A–E of striata obtained from a control mouse. The vertical lines indicate the retention times of the analytes.

## 6.2. In vivo experiments and results

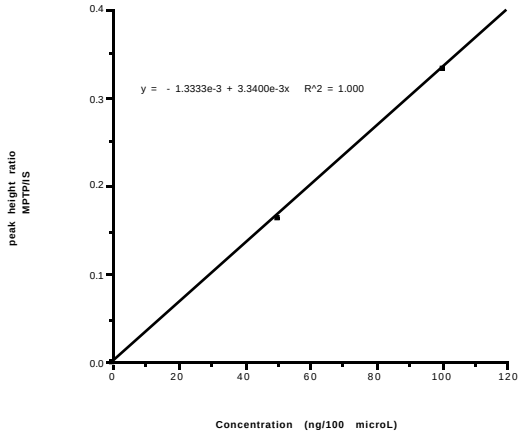
To examine the MAO-B inhibition properties of 7-NI in the C57Bl/6 mouse model an LC-DA assay was developed.[234] With this assay it is possible to monitor all four compounds of interest, MPTP, $MPDP^+$, $MPP^+$ and 7-NI. Differences in the retention time and chromophores made it possible to monitor each compound on a different channel without interference from other peaks. HPLC analyses were performed on a Hewlett Packard 1100 HPLC system equipped with a UV/VIS diode array detector. The mobile phase (pH 4.70) consisted of 70% milli Q water containing 0.6% (v:v) glacial acetic acid (solvent A) plus 1.0% (v:v) TEA and 30% acetonitrile. For quantitative analyses the compounds were monitored at the following wavelengths: 244 nm (MPTP), 285 nm ($MPP^+$), 345 nm ($MPDP^+$), 360 nm (7-NI) and 390 nm for 1-cyclopropyl-4-(1-methylpyrrol-2-yl)pyridinium iodide [**167**, internal standard (IS)].

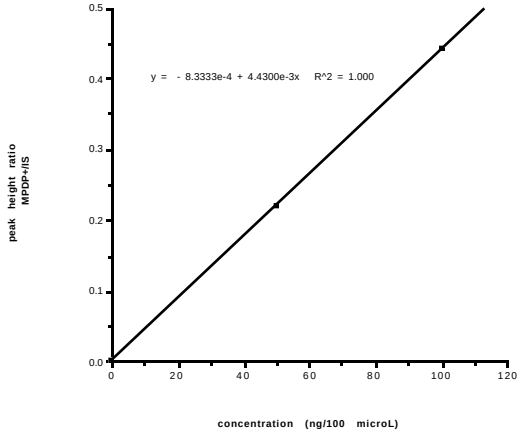


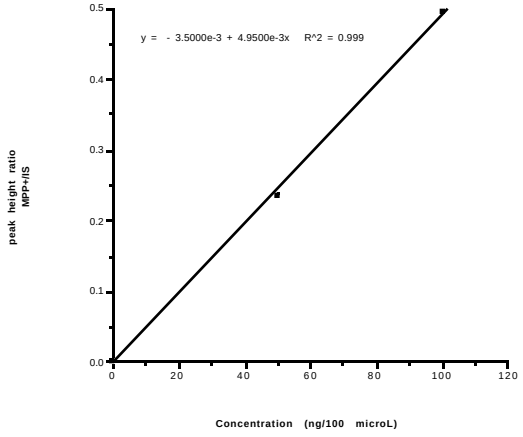
**167**

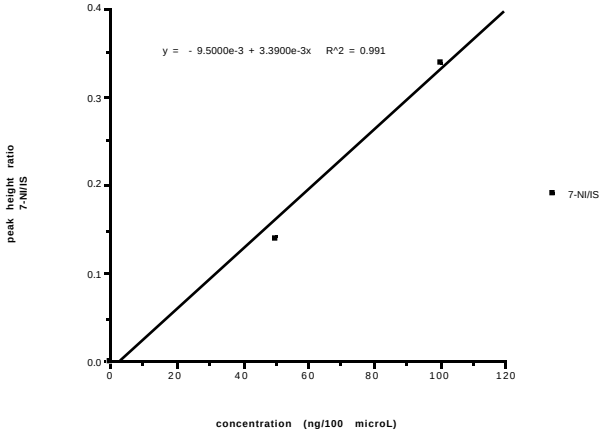
The levels of MPTP, $MPDP^+$, $MPP^+$, and 7-NI were calculated from calibration curves which were prepared by injecting 3 calibration standards containing all four analytes in triplicate (100 μL), made fresh for every new curve. The calibration standards were prepared in 90% solvent A:10%

acetonitrile. The concentration of IS was 0.060 μM in each standard and the peak-height ratios of the analytes/IS were plotted versus the pmoles of analytes. The pyrrolylpyridinium IS **167**, a compound with similar physicochemical properties to those of $MPP^+$, gave standard calibration curves with good linearity ($r^2$ = 0.997 to 1.000) over the relevant concentration ranges for all 4 analytes as illustrated below.



Quantitative estimations were calculated by comparing the peak-height ratios of the analyte/IS of the sample versus the peak-height ratios of the calibration curves.

To ensure that the assay was effectively recovering the analytes adequately from the brain tissue, the recovery of analytes was examined utilizing two sets of samples. In the first set (group 1, n = 3) 120 ng of each analyte and 11.5 ng of IS in 90% solvent A:10% acetonitrile were added to 20 mg mouse brain tissue (total volume 240 μL). For group 2 (n = 3) the same procedure was followed, only the IS was not added. The tissue samples were then homogenized and allowed to stand 30 min on ice. After centrifugation (5 min at 16000 g), 180 μL aliquots of the supernatant were transferred to new tubes. To group 2, IS was added (8.6 ng in 10 μL) and to group 1, an equivalent volume of solvent was added yielding the same final concentration of IS (0.139 μM) in both groups. Aliquots of 100 μL were injected for analysis. Recovery was calculated by Average [analyte]group1/Average [analyte]group 2 x 100%. The results are summarized in Table 27.

**Table 27**. Summary of the Analyte Recovery Experiment, Analyte Tissue Binding Experiment, and Analyte Extraction Experiment

| Analytes | | MPTP | $MPDP^+$ | $MPP^+$ | 7-NI |
|---|---|---|---|---|---|
| Recovery exp. | | 97.3% | 94.6% | 94.0% | 92.7% |
| Tissue binding exp. | A[a] | 101.9% | 99.6% | 100.8% | 88.5% |
| | B[a] | 81.2% | 100.4% | 108.2% | ND[c] |
| Extraction exp.[b] | | 97.4% | 93.5% | 106.5% | --- |

[a] MPTP, $MPDP^+$, $MPP^+$, 7-NI A = 4.80, 3.70, 3.40, and 6.15 μM
B = 0.48, 0.37, 0.34, and 0.62 μM

[b] The percentages were calculated by Average [analyte]method B/Average [analyte]method A x 100% (for a description of the methods see experimental section).

[c] ND = not detectable.

To determine the amount of the metabolites that bind to the brain tissue, a method described previously by Schinka *et al* was used.[260] For group 1 (n =3 ), 400 μL of a 12.5% brain homogenate in 0.32 M sucrose was added to 100 μL of 0.32 M sucrose containing the analytes (MPTP, $MPDP^+$, $MPP^+$ and 7-NI). In group 2 (n = 3), 400 μL of 0.32 M sucrose alone was added to 100 μL 0.32 M sucrose containing the analytes (MPTP, $MPDP^+$, $MPP^+$ and 7-NI) were added. Two experiments were performed, one experiment involved high concentrations of the metabolites (experiment A) and the second was carried out at low metabolite concentrations (experiment B). The final concentrations of

the analytes were the following:

- Experiment A: 4.80 μM, 3.70 μM, 3.40 μM, 6.15 μM for MPTP, $MPDP^+$, $MPP^+$ and 7-NI, respectively.
- Experiment B: 0.48 μM, 0.37 μM, 0.34 μM, 0.62 μM for MPTP, $MPDP^+$, $MPP^+$ and 7-NI, respectively.

The homogenate was vortexed for 1 min and to each tube 500 μL 90% solvent A:10% acetonitrile with IS was added. After the samples were vortexed for 2 min, they were allowed to stand 1 hour on ice. After centrifuging (5 min at 16000 g) the supernatant was filtered through a 13 mm nylon syringe filter (0.45 mm, Alltech Associates Inc., Deerfield, IL). Aliquots of 100 μL were injected for analysis. Percentage tissue binding was calculated by Average [analyte]brain / Average [analyte]sucrose x 100%. Statistical comparisons were calculated using InStat, GraphPad Software using the student t-test. The results are summarized in Table 27.

To compare the extraction procedure that is used in this assay and the more established method using 5% TCA,[261] the following experiment was carried out. Mice (n=10) were injected i.p. with 143 μmol/Kg MPTP·HCl and sacrificed 25 min later. The striatal tissue of 5 mice was processed further as described above (method A), the other five were processed using 5% (w:v) TCA instead of 90% solvent A:10% acetonitrile (method B). Because TCA caused a peak in the chromatogram that interfered with the peaks of some analytes with the reversed phase column, these analyses were performed with an Adsorbosphere SCX (5mm, 250 x 4.6 mm; Alltech Associates Inc., Deerfield, IL) column. Calibration curves were made in both 5% TCA and 90% solvent A:10% acetonitrile. The results are summarized in Table 27.

With the very reliable LC-DA assay, we performed in vivo experiments in the C57Bl/6 mouse model for MPTP neurotoxicity that were designed to evaluate the effects of 7-NI on the metabolism of MPTP in vivo (see experimental section for animal handling).

On the first day of the first series of experiments, mice were injected s.c. with 0, 137, 273 or 546 μmol/Kg 7-NI in peanut oil. This was followed 30 min later by an i.p. injection with 238 μmol/Kg. The animals were subsequently sacrificed by cervical dislocation at 90 min post MPTP treatment. The brains were removed and the striata were dissected on ice, placed in an Eppendorf tube, weighed and 120 μL containing 0.065 μM IS in 90% solvent A:10% acetonitrile were added. The samples were homogenized and centrifuged for 5 min at 16000 g, yielding a final concentration of 0.060 μM IS. For each sample, a 100 μL aliquot of the supernatant was injected onto the HPLC for quantitative analysis. The results are summarized in Table 28.

**Table 28**. Striatal $MPP^+$ and 7-NI Concentrations in MPTP (238 μmol/Kg) Treated C57Bl/6 Mice Pretreated with Increasing Doses of 7-NI.

| Number of mice | Dose 7-NI (mmol/Kg)[a] | 7-NI (pmol/mg wet tissue ± SD) | $MPP^+$ (pmol/mg wet tissue ± SD) | Percent $MPP^+$ |
|---|---|---|---|---|
| 4 | 0 | ND[b] | 49.0 ± 8.0 | 100.0 |
| 5 | 137 | 3.3 ± 1.3 | 37.6 ± 10.6 | 76.7 |
| 4 | 273 | 12.9 ± 2.3 | 26.9 ± 2.4[c] | 54.9 |
| 4 | 546 | 32.8 ± 9.2 | 24.2 ± 5.2[c] | 49.4 |

[a]Administered s.c. 30 min prior to i.p. [b]ND = Not detectable. [c]Significantly different from no 7-NI pretreatment ($p < 0.005$).

In the second series of experiments, mice were pretreated with peanut oil containing 0 [controls (C)] or 273 μmol/Kg [treated (T)] 7-NI followed 30 min later by i.p. MPTP·HCl (87, 115 or 238 μmol/Kg). The animals were sacrificed 90 min after the MPTP administration and the brains were further processed as described above. The results are summarized in Table 29.

**Table 29**. Striatal $MPP^+$ Concentrations in C57Bl/6 Mice Pretreated s.c. with 273 μmol/Kg 7-NI Prior to Increasing Doses of i.p. MPTP.

| # of animals (C, T) | Dose MPTP (μmol/Kg) | [$MPP^+$] (C) pmol/mg wet tissue ± SD | [$MPP^+$] (T) pmol/mg wet tissue ± SD | % $MPP^+$ (T/C) |
|---|---|---|---|---|
| 4,4 | 87 | 5.9 ± 0.5 | 3.2 ± 0.3 | 53.4[b] |
| 6,6 | 115 | 14.1 ± 3.3 | 9.5 ± 1.4 | 67.1[a] |
| 4,4 | 238 | 55.4 ± 6.4 | 33.8 ± 3.2 | 61.0[a] |

[a]Significantly different T from C, $p < 0.0005$. [b]Significantly different T from C, $p < 0.05$.

In the third set of experiments a pharmacokinetic profile of MPTP metabolism was obtained. The concentration of 7-NI and MPTP were held constant, but the time of sacrifice was varied. Mice were injected s.c. with peanut oil containing 0 (C) or 273 μmol/Kg (T) 7-NI followed 30 min later with 238 μmol/Kg MPTP·HCl i.p. and were subsequently sacrificed at 10, 30, 90, 120 and 240 min post MPTP treatment. The obtained tissues were processed in the same manner as described above. The results of these studies are

summarized in Table 30a for control mice and 30b for treated mice.

**Table 30a**. Striatal MPTP, MPDP+, MPP+, and 7NI Concentrations in C57Bl/6 Control Mice

| | | Controls (C) Striatal concentration in pmoles per mg wet tissue ± SD | | |
|---|---|---|---|---|
| Number (C,T) | Time (min)[a] | MPTP | $MPDP^+$ | $MPP^+$ |
| 4,4 | 10 | 134.4 ± 21.2 | 38.0 ± 6.6 | 11.7 ± 3.2 |
| | | [S ($MPDP^+$ + $MPP^+$) = 49.7 ± 9.1] | | |
| 6,5 | 30 | 10.8 ± 4.0 | 13.5 ± 3.9 | 38.1 ± 6.9 |
| | | [S ($MPDP^+$ + $MPP^+$) = 51.5 ± 10.2] | | |
| 4,4 | 90 | ND[d] | ND | 55.4 ± 6.4 |
| 6,6 | 120 | ND | ND | 32.0 ± 3.4 |
| 6,5 | 240 | ND | ND | 31.1 ± 5.1 |

[a]: Time in minutes is post-MPTP injection.

[b]: Significantly different T from C, $p < 0.05$.

[c]: Significantly different T from C, $p < 0.005$.

[d]: Not detectable.

**Table 30b**. Striatal MPTP, MPDP+, MPP+, and 7NI Concentrations in C57Bl/6 Treated Mice

| | | Treated (T) Striatal concentration in pmoles per mg wet tissue ± SD | | | Ratio % Metabolites (T/C) |
|---|---|---|---|---|---|
| Number (C,T) | Time (min)[a] | MPTP | $MPDP^+$ | $MPP^+$ | |
| 4,4 | 10 | 233.3 ± 66.5[b] | 27.9 ± 7.0 | 9.2 ± 3.3 | |
| | | [S ($MPDP^+$ + $MPP^+$) = 37.1 ± 7.1] | | | |
| | | | | | 74.7 |
| 6,5 | 30 | 30.2 ± 11.8[c] | 12.2 ± 2.7 | 23.7 ±.4.9 | |
| | | [S ($MPDP^+$ + $MPP^+$) = 35.8 ± 7.2] | | | |
| | | | | | 69.5[b] |
| 4,4 | 90 | 3.2 ± 3.3 | ND | 33.8 ± 3.2 | 61.0[c] |
| 6,6 | 120 | ND | ND | 22.4 ± 2.2 | 71.5[c] |
| 6,5 | 240 | ND | ND | 18.4 ± 3.6 | 59.2[c] |

[a]: Time in minutes is post-MPTP injection.

[b]: Significantly different T from C, $p < 0.05$.

[c]: Significantly different T from C, $p < 0.005$.

[d]: Not detectable.

In order to determine the levels of 7-NI in the mouse brain and to gain a better insight into the absorption profile of the 7-NI in peanut oil at early time points, 7-NI was injected s.c. in peanut oil containing 273 μmol/Kg 7-NI and subsequently the mice were sacrificed at 7, 15 and 25 min post injection. The

obtained striata were processed as previously described. The data are summarized in Table 31. At 7 min (post 7-NI) the concentration of 7-NI was 8.4 pmol/mg tissue and was rising. From 10 min to 40 min the concentration of 7-NI was constant and the maximum level was found at 60 min. Subsequently 7-NI was cleared slowly from the brain.

**Table 31.** C57Bl/6 Mice (n=6) Treated with 7-NI in Peanut Oil (s.c., 273 μmol/Kg) and Sacrificed at the Indicated Time Post Injection.

| Time (min) | [7-NI] (pmol/mg tissue ± SD) |
|---|---|
| 7 | 8.4 ± 1.8 |
| 15 | 10.3 ± 1.9 |
| 25 | 11.3 ± 2.5 |

Thus the data (see below) demonstrates that 7-NI inhibits MAO-B and affects the metabolism of MPTP in vivo. The next question to be answered was whether or not the 7-NI inhibition of MAO-B was responsible for the reported neuroprotection against MPTP neurotoxicity[227] in C57Bl/6 mice or if the inhibition of nNOS contributes to the observed neuroprotection. In order to begin to address these questions, another series of experiments was designed. Dopamine and DOPAC levels were measured by HPLC with electrochemical detection (see Chapter 4 for sample processing and analysis) in C57Bl/6 mice which received MPTP (238 μmol/Kg) 30 minutes following pretreatment. The groups A-D were pretreated differently. Group A, the controls, received peanut oil only at t = 0 min and saline at t = 30 min. Group B received (R)-deprenyl

(11.2 μmol/Kg) at t = 0 min and MPTP (238 μmol/Kg) at t = 30 min. Group C received peanut oil only at t = 0 min and MPTP (238 μmol/Kg) at t = 30 min. Group D received 7-NI (273 μmol/Kg) at t = 0 min and MPTP (238 μmol/Kg) 30 min following pretreatment. The results are summarized in Table 32. In this experiment, the behavior of the animals after MPTP administration was noted. As previously observed, at lower dosages of MPTP, mice behaved normally and were somewhat active directly after the injection. At the highest dose of MPTP however, almost immediately after MPTP injection the mice hardly moved and appeared to be sick. They developed a very stiff tail which pointed straight backwards (Straub tail) and they start to shake and shiver. Ten min post injection they went through a phase of shaking and involuntary movements like moving backwards, falling, etc. After 30 min this behavior stopped and the animals were depressed again, not showing much movement at all, breathing rapidly and shallowly in the beginning. Mortality occured mostly during the first 20 - 30 min phase. If the animals survived this period, the survival rate increased. Animals pretreated with 7-NI displayed greater activity and a lower percentage appeared disturbed by the MPTP injection.

**Table 32**. Striatal Dopamine and DOPAC Concentrations in C57Bl/6 Mice (n=9) After Treatment as Indicated.

| Group[a] | [Dopamine] (pmol/mg tissue) | [DOPAC] (pmol/mg tissue) | % Dopamine of controls | % DOPAC of controls |
|---|---|---|---|---|
| A | 112 ± 11.6 | 11.9 ± 2.9 | 100 | 100 |
| B | 109.2 ± 4.2 | 10.5 ± 1.7 | 97.3 | 88.2 |
| C | 20.7 ± 17.9[b] | 3.0 ± 2.9[b] | 18.4[b] | 25.2[b] |
| D | 44.3 ± 15.6[b,c] | 4.9 ± 2.0[b] | 39.5[b,c] | 41.2[b] |

[a] Group A: peanut oil, saline; group B: R-deprenyl (11.2 μmol/Kg), MPTP (238 μmol/Kg); group C: peanut oil, MPTP (238 μmol/Kg); group D: 7-NI (273 μmol/Kg), MPTP (238 μmol/Kg).
[b] Significant different from control ($p<0.0001$).
[c] Significant different from group C ($p=0.0115$).

The results (Table 32) show a dramatic decrease in striatal dopamine and DOPAC levels after MPTP administration only. This is consistent with previous observations. Pretreatment with (R)-deprenyl prevents MPTP induced dopamine depletion and pretreatment with a single s.c. 7-NI injection also shows protection against dopamine and DOPAC depletion although 7-NI is not as neuroprotective as is deprenyl. This in part may be due to the fact that (R)-deprenyl is an irreversible inhibitor while 7-NI is only a competitive inhibitor. The concentration of dopamine is significantly different from both controls and MPTP treated animals, so toxicity is partially attenuated by a single 7-NI injection.

Based on the results obtained (Table 32) 7-NI does provide some protection against MPTP toxicity. Several experiments were designed with the

intent of trying to determine if the observed 7-NI neuroprotection against MPTP toxicity is a result of MAO-B inhibition, resulting in less metabolism of MPTP to $MPP^+$ and therefore less observable toxicity, or if the neuroprotection is a result of the inhibition of nNOS which would result in less NO formation which is proposed to be involved in the mediation of cell death. In experiment 1, group 1, the controls received only sterile saline. Group 2 animals were treated i.p. with 238 μmol/Kg MPTP·HCl only. From the kinetic profiles, we know that most of the MPTP is metabolized by approximately 25 minutes after dosing with MPTP. Based on these results we decided to dose groups 3 and 4 in the following manner: Group 3 was given 238 μmol/Kg MPTP·HCl i.p. at t = 0 and 25 minutes post MPTP injection, the group 3 animals received s.c. 273 μmol/Kg 7-NI. Group 4 received a s.c. injection of 273 μmol/Kg 7-NI at t = 0, and at 30 min post 7-NI treatment, 238 μmol/Kg MPTP·HCl was given. The animals were sacrificed 8 days after treatment and the samples were processed and analyzed by HPLC as described in chapter 4. The data are summarized in Table 33.

**Table 33**. The Evaluation of the Role of 7-NI in MPTP Induced Neurotoxicity (Experiment 1)

| Group ID (# of mice) | Treatment | % DA of controls |
|---|---|---|
| 1 (9) | no drugs | 100 |
| 2 (9) | MPTP only | 12.4 |
| 3 (9) | t = 0 min MPTP<br>t = 25 min 7-NI | 22.5 |
| 4 (9) | t = 0 min 7-NI<br>t = 30 min MPTP | 28.1 |

From experiment 1, we note that the pretreated 7-NI group 4 and the post treated 7-NI group 3 display a very similar degree of neuroprotection against MPTP toxicity.

To determine the levels of $MPP^+$ in the brains of mice treated with MPTP, the following experiments were performed. C57Bl/6 mice were treated with 238 μmol/Kg MPTP·HCl and group 1 was sacrificed at 4 hours post MPTP treatment. Group 2 was sacrificed 8 hours after treatment and group 3 was sacrificed 24 hours after treatment. Table 34 summarizes the results.

**Table 34**. The $MPP^+$ Levels in C57Bl/6 Mice Treated with 238 μmol/Kg MPTP·HCl and Sacrificed at 4, 8, and 24 hrs.

| Group ID | Time of sacrifice (hrs) | $MPP^+$ levels pmol/mg striata |
|---|---|---|
| 1 | 4 | 28.8 |
| 2 | 8 | 18.9 |
| 3 | 24 | 1.0 |

Since $MPP^+$ is present in the brain for 24 hours after in vivo treatment of MPTP, we designed an experiment in which 7NI levels are maintained for a longer period of time. With this longer treatment with 7-NI we anticipated that we would see a combined effect on MPTP neuroprotection, some contribution to neuroprotection from pretreatment with 7NI causing MAO-B inhibition (less metabolism of MPTP) and some contribution from post 7-NI treatment which should, in principal, inhibit nNOS and reduce the formation of NO. The neurotoxicity experiment 2, contained 6 groups A-F. The treatment of groups A-F are listed below:

- Group A animals, the controls, received only saline or peanut oil.
- Group B animals received 238 μmol/Kg MPTP·HCl (50 mg/Kg) only
- Group C animals were given 191 μmol/Kg MPTP·HCl (40 mg/Kg) only
- Group D animals were pretreated at t = 0 min with 273 μmol/Kg 7-NI followed by an i.p. injection of 238 μmol/Kg MPTP·HCl.
- Group E animals were pretreated with 273 μmol/Kg 7-NI at t=0 min and at t=30 min 238 μmol/Kg MPTP·HCl was given. At 8 hour intervals for 4 days

post MPTP treatment, 273 μmol/Kg 7-NI was given.

- Group F were given 238 μmol/Kg MPTP·HCl at t = 0 min and at 30 min post MPTP the selective inhibitor (R)-deprenyl (10 mg/Kg) was given.

The animals were sacrificed on day 8 and the samples were analyzed by HPLC with electrochemical detection. Group D, in principal, should give us the component of neuroprotection due to MAO-B inhibition. Group E should give the contribution due to MAO-B inhibition and the putative contribution from nNOS inhibition.

**Table 35**. The Evaluation of the Contributing Components of 7-NI Neuroprotection Against MPTP Toxicity (Experiment 2)

| number of animals | Group ID | DA pmol/mg striata | % DA of controls |
|---|---|---|---|
| 8 | A- controls | 113.01 | 100 |
| 4 | B- MPTP (50 mg/Kg) | 30.51 | 27 |
| 8 | C- MPTP (40 mg/Kg) | 51.59 | 45.7 |
| 6 | D- pre 7-NI | 77.33 | 68.4 |
| 8 | E- Pre/ post 7-NI | 75.00 | 66.4 |
| 5 | F- post deprenyl | 83.47 | 73.9 |

With experiment 2, (Table 35), the group D animals which were pretreated with 7-NI, and group E animals, which were given pre and post treatments with 7-NI, show virtually the same neuroprotection against MPTP toxicity. Group F

animals, which received post treatment with deprenyl 30 minutes after MPTP, show very good protection against MPTP toxicity. The normal protocol to obtain protection against MPTP toxicity is to pretreat with (R)-deprenyl but here we observe some protection with post treatment as well. It has been reported that deprenyl also protects against $MPP^+$ toxicity.[262-265] This may also apply with 7-NI. We must note that keeping 7-NI levels high in the mice in group E did not increase neuroprotection which follows the in vivo studies previously described (Table 28) in which we see a leveling off of 7-NI effects. The results obtained with groups D-F lead to the initial conclusion that the neuroprotection observed against MPTP toxicity is solely due to MAO-B inhibition and not nNOS inhibition as reported.[227,228]

## 6.3 Discussion

The data obtained from the recovery experiment showed good recoveries for all compounds (Table 26). 7-NI had the lowest recovery of 92.7%, $MPP^+$, $MPDP^+$, and MPTP had a recoveries of 94.0%, 94.6%, and 97.3%, respectively. Also in the tissue binding experiment the numbers were good. At the concentration of 100 ng of all analytes per sample, the recoveries were 101.9% (MPTP), 99.6% ($MPDP^+$), 100.8% ($MPP^+$) and 88.5% (7-NI). At the low concentration (10 ng per sample), only the MPTP recovery was found to be lower. 7-NI was not quantitatively detectable at this low concentration which defines the limit of detection. A comparison of the method used to process the tissue samples obtained with the more commonly used method involving 5% TCA showed no significant difference. The concentrations found in the striatal

tissue processed by both methods were similar (Table 27).

The MAO-B competitive inhibitor properties of 7-NI prompted us to examine the influence of 7-NI on the levels of $MPP^+$ in the striata of MPTP treated C57Bl/6 mice. An LC-DA assay was developed to provide quantitative estimations of the striatal concentrations of all 4 compounds of interest (MPTP, $MPDP^+$, $MPP^+$ and 7-NI) which was used to evaluate the levels of metabolites in the experiments discussed. The DA depletion experiments used the HPLC assay with electrochemical detection as described in Chapter 4.

The first series of experiments examined the effects on MPTP metabolism at different doses of 7-NI (0–546 μmol/Kg) administered 30 min prior to MPTP (238 μmol/Kg) with sacrifice 90 min post-MPTP. At this time point, which is reported to coincide with the maximum striatal $MPP^+$ levels[227] only $MPP^+$ and 7-NI were detected in the striatal extracts. The results (Table 28) show that, as the dose of 7-NI increases, the concentration of striatal 7-NI increases and the concentration of $MPP^+$ decreases. At the reported maximally neuroprotective 7-NI dose in mice (273 μmol/Kg), the striatal concentration of $MPP^+$ is only 54.9% of the control value. There is an apparent leveling of this effect since a dose of 546 μmol/Kg 7-NI did not result in a much greater decrease in $MPP^+$ concentration even though the concentration of 7-NI was considerably higher. A more complete pharmacokinetic profile will be required to establish the relationship between striatal 7-NI concentration and the rate of conversion of MPTP to $MPP^+$. The half-life of MPTP in the striatum is likely to be much shorter than that of 7-NI under these experimental conditions. Consequently, a more meaningful estimation of the relationships between 7-NI concentrations and the rate of $MPP^+$ formation might be found at an earlier time

point.

The second series of experiments examined the effects of a fixed s.c. dose of 7-NI on the metabolism of varying i.p. doses of MPTP. The animals were pretreated with 273 μmol/Kg 7-NI 30 min prior to single MPTP doses ranging from 87 to 238 μmol/Kg with sacrifice at 90 min post-MPTP treatment. The results (Table 29) demonstrate that the striatal $MPP^+$ concentrations are significantly lower in the mice pretreated with 7-NI (53.4–67.1% $MPP^+$ T/C) at all doses of MPTP. This establishes that 7-NI inhibits MAO-B in vivo and influences the metabolism of MPTP by retarding its conversion to $MPP^+$.

The third series of experiments was designed to evaluate the time course of the effects of 7-NI on the metabolic profile of MPTP in the striatum. We chose a single, neurotoxic dose of MPTP (238 μmol/Kg) and a dose of 7-NI (273 μmol/Kg) that is reported to afford the maximum protection when administered 30 min prior to MPTP.[227] Animals were sacrificed at 10–240 min post-MPTP treatment.

The early time points examined in these studies provide information on the striatal concentrations of MPTP and $MPDP^+$ in addition to 7-NI and $MPP^+$ (Table 30a and 30b). In general, the striatal half-life of MPTP (i.p. administration) is quite short relative to that of 7-NI (s.c. administration) and $MPP^+$. If inhibition of MAO-B by 7-NI is of toxicological importance, such pharmacokinetic variables could impact on 7-NI's neuroprotection against MPTP toxicity. Consistent with the MAO-B inhibiting properties of 7-NI observed in vitro, the striatal concentrations of MPTP in the treated animals at 10 and 30 min are 174% and 280%, respectively, of the corresponding levels for the untreated animals. Unlike the control animals, MPTP is still detectable in the 7-

NI treated animals at 90 min. Even though the MPTP levels are higher in the 7-NI treated animals, the extent to which MPTP is converted to $MPDP^+$ and $MPP^+$ is significantly lower at these early time points. This shift in the metabolism of MPTP is similar to, although less marked than, that reported when animals are pretreated with pargyline, a potent MAO mechanism based inactivator[266]

Experiments 1 and 2 (Table 33 and 35) were designed to evaluate what percentage of 7-NI protection against MPTP toxicity is due to MAO-B inhibition and what percentage is due to nNOS inhibition. The preliminary results point to the possibility that 7-NI may exhibit its neuroprotection solely by MAO-B inhibition. The interpretation of the biological results using 7-NI become difficult due to the complexity of the many synergistic factors that may be involved. We are currently working to design in vivo experiments that may begin to answer the questions surrounding the mechanism of 7-NI neuroprotection against MPTP toxicity in vivo.

In summary, the results of these studies demonstrate that 7-NI inhibits the MAO-B catalyzed oxidation of MPTP and decreases the striatal levels of $MPP^+$ in MPTP treated mice. Treatment with the MAO-B mechanism based inactivator (R)-deprenyl also blocks the conversion of MPTP to $MPP^+$ in the brain [267]and protects against MPTP's neurotoxic properties.[45,268] Also of interest, however, are reports that (R)-deprenyl also may protect against $MPP^+$ toxicity.[262-265] These factors may be important in the interpretation of data and designing experiments to answer the questions surrounding 7-NI's potential neuroprotection. The extent to which 7-NI's inhibition of MAO-B may contribute to 7-NI's neuroprotecting properties in MPTP treated C57Bl/6 mouse are currently being examined further in our group.