Possible Environmental Risk Factors Associated with Parkinson's Disease

[redacted]

Decreased Risk

Exposure to toxins

- Pesticides
- Consumption of well water
- Pollution from industrial plants
- Diet - consumption of >7 portions of tropical fruit daily

Parkinson's Disease

Cigarette smoking

- Nicotine effects? (nicotinic receptors)

Coffee drinking

- Caffeine effects? (adenosine A_2 antagonist)

Trauma

- Head injury (boxing)

Infection

- Bacterial (*Nocardia asteroides*) or viral (influenza)

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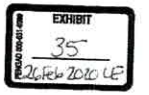
SYNG-PQ-00493396

Investigating the Nigrostriatal Toxicity of Paraquat Dichloride

Dr Louise Marks,
Syngenta Health Assessment, Alderley Park



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Study Design

- 8-10 week old male $C_{57}Bl_{6J}$ mice
- 'in-life' phase - mice received i.p. injections of:

  sterile saline (control group)

  10 mg/kg paraquat dichloride once or twice a week for 3 weeks

  20 mg/kg MPTP daily for 10 days
- n of 5-9 per group
- animals killed 7 days after the final dose

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SYNG-PQ-00493397

Parkinson's Disease - What is it?

- Parkinson's disease is a neurodegenerative disease caused by dopamine deficiency in the **striatum**, and loss of dopaminergic neurones in the **substantia nigra.**
- Symptoms include tremors, muscle rigidity, involuntary movements and postural changes.
- Affects approximately 1 in 500 of the UK population > 65 years.
- Mean age for onset of symptoms is typically between 60-65 years old, and only 5% of Parkinson's patients develop symptoms before age 40.
- No clear genetic component to idiopathic Parkinson's disease, except in some cases of early-onset.
- "Environmental factors" may play a role in the aetiology of the disease?

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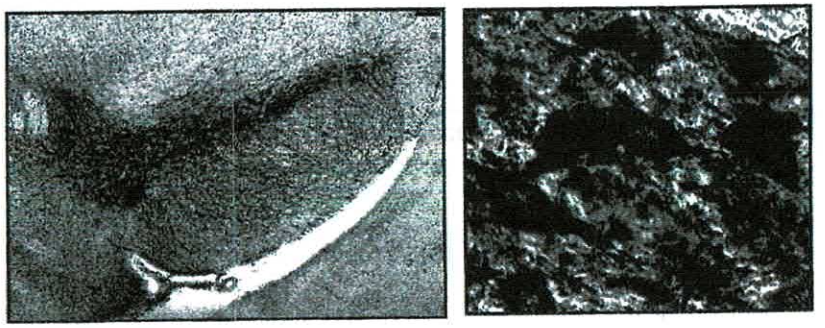
SYNG-PQ-00493395

Methodology - Endpoints

Investigating cell loss in SNpc



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SYNG-PQ-00493400

Methodology - Endpoints

Analysis of Striatal Dopamine content

- HPLC –EC was used to determine the concentrations of dopamine (DA) and its key metabolites in each sample.

Behavioural analysis

- Clinical observations were performed for 2-3 hours post dosing.
- Locomotor activity was assessed using automated activity chambers at 2, 24, 48 hours and 7 days after the final dose.

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SYNG-PQ-00493398

What is Stereology?

- 'Method of recreating or estimating geometric properties of an object in space'
  - **number**, volume, surface, length
- Old methods of cell counting - accuracy debatable
- New design based methods much more accurate - 3D

- **OPTICAL FRACTIONATOR**
  - unbiased method of estimating cell number
  - representative sampling

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SYNG-PQ-00493401

Methodology - Endpoints

Investigating cell loss in SNpc

- Mid/hindbrain blocks were removed and immersion fixed in 4% paraformaldehyde and cryoprotected in graded sucrose prior to cutting 40 μm sections on a freezing sledge microtome.
- Free floating immunohistochemistry using a tyrosine hydroxylase (TH) antibody (*Pel Freez;1:4000*) was carried out on every 3rd section.
- Adjacent sections were stained with cresyl violet (CV) for determination of total cell counts.
- TH^+ and CV^- cells were counted in the substantia nigra pars compacta using the optical fractionator method of stereology.

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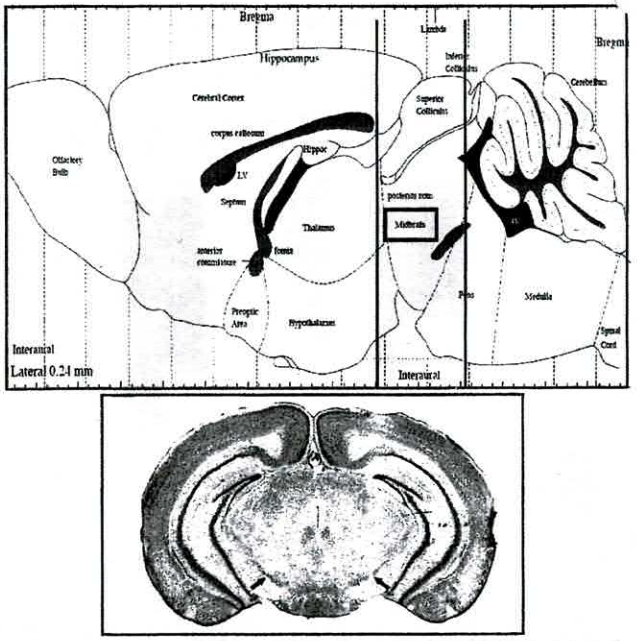
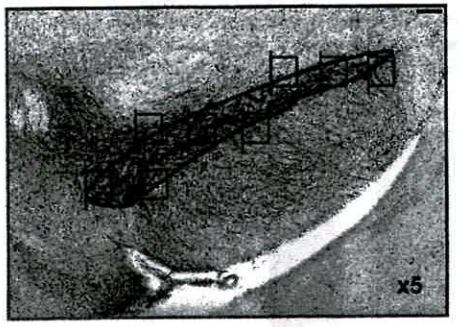
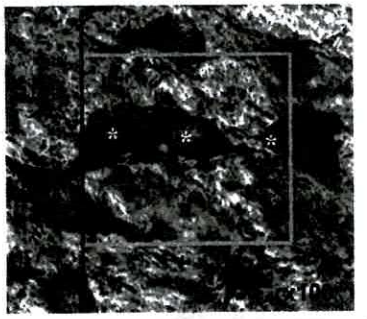
-PQ-00493399



SYNG-PQ-00493402

The Optical Fractionator Method of Stereology





$$N = \text{Sum } Q^{-} \times 1/\text{ssf} \times 1/\text{asf} \times 1/\text{tsf}$$

SYNG-PQ-00493403

SYNG-PQ-00493404

Replication of studies from Parkinson's Institute

- Initial cell count data did not produce a statistically significant reduction in dopaminergic cell number in the SNpc
- We needed to understand where differences may lie in terms of the stereology methodology
- From discussions with Di Monte's group at the Parkinson's Institute we gained insight into the stereology methodology and highlighted differences in the set ups being used in our two labs

equipment used – Automated stage + MicroBrightfield software
delineation of SNpc
exact parameters used

SYNG-PQ-00493405

Study 1- Effect of MPTP Paraquat on GFAP expression

- The number of reactive astrocytes has been reported to increase following exposure to both MPTP and Paraquat (Vila *et al.*, 2001; Kurkowska-Jastrzebska *et al.*,1999; Thiruchelvam *et al.*,2000; Mcormack *et al.*,2002)
- IHC using the astrocytic marker GFAP labelled astrocytes throughout the brain- hippocampus, SNpc, SNr
- Administration of 3 x 10mg/kg PQ did not appear to alter the pattern or intensity of astrocytic staining in the SNpc

- **Western blots could be carried out in an attempt to quantify the levels of GFAP expression in the brain following PQ administration**

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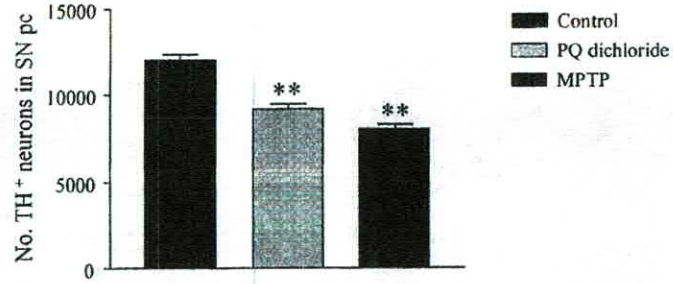
SYNG-PQ-00493408

Study 1- Effects of Dosing Paraquat Once a Week for 3 weeks

TH^+ cell counts in SNpc



Data represents mean ± SEM. Saline control group received 3 weekly i.p. injections of sterile saline, n=9; Treated group received 3 weekly injections of 10 mg/kg PQ dichloride, n=9; MPTP group received 10 daily injections of 20 mg/kg MPTP, n=9. Values analysed by one way ANOVA followed by Newman-Keuls post test **p<0.001

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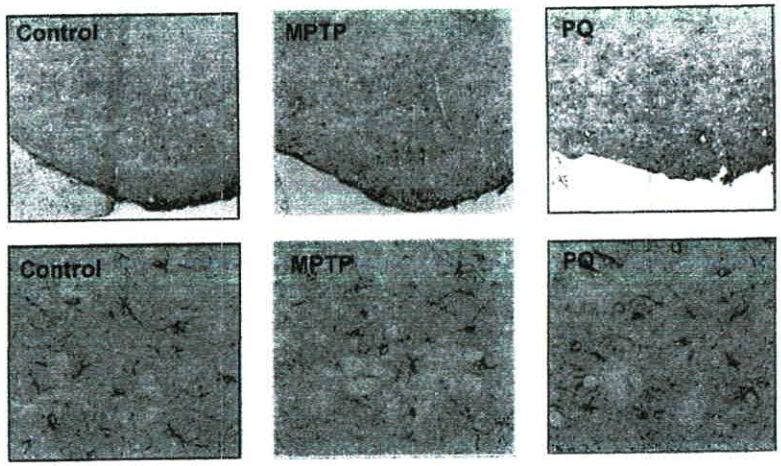
SYNG-PQ-00493406

Effect of MPTP and Paraquat on GFAP expression



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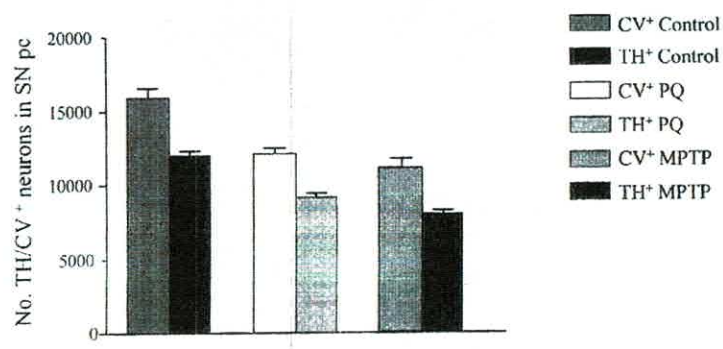
SYNG-PQ-00493409

Study 1- Effects of Dosing PQ 1x / week for 3 weeks

CV^+ cell counts in SNpc

Using modified stereology parameters and new set up – 24% ↓ CV^+ cells (PQ) : 31% ↓ CV^+ cells (MPTP):



Data represents mean ± SEM. Saline control group received 3 weekly i.p. injections of sterile saline, n= 8 (CV), 9 (TH); PQ group received 3 weekly injections of 10mg/kg PQ dichloride, n=9 : MPTP group received 10 daily injections of 20mg/kg MPTP, n=9.

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3-PQ-00493407

Study 1- Haematoxylin and Eosin Staining

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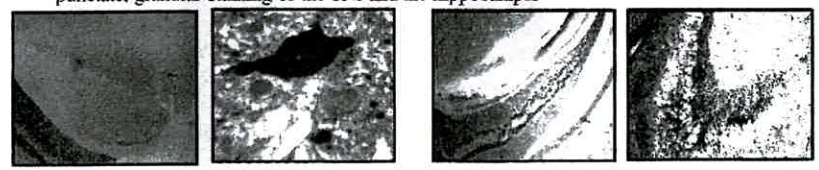
Study 1- Effect of MPTP and Paraquat on α-synuclein expression

- Alpha synuclein expression has been reported to be up regulated following exposure to both MPTP

  and Paraquat (Vila *et al.*,2000; McCormack *et al.*,2002; McCormack *et al.*,2003)
- IHC using the Syn-1 antibody for alpha synuclein produced two main patterns of staining:
  - intracellular cytosolic and nuclear staining of neurons in the SNpc and VT
  - punctate, granular staining of the SNr and the hippocampus



- Administration of 3 x 10mg/kg PQ did not appear to alter the pattern or intensity of alpha synuclein

  staining in the SNpc. however the intensity of the granular staining in the SNr did appear to be greater in

  animals exposed to MPTP and PQ
- Western blots could be carried out in an attempt to quantify the levels of alpha synuclein in

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Study 1 – Initial replication of studies from Parkinson's Institute

Conclusion

- Dosing with 10 mg/kg paraquat dichloride once a week for 3 weeks resulted in a statistically significant reduction in cell number in the SNpc *(24%)*
- No statistically significant change in the concentration of striatal DA or DA turnover was observed
- The absolute values for TH^+ and CV^+ cell counts and the % reductions are comparable with what has been reported by the group at the Parkinson's Institute
- No major differences were observed across groups in GFAP, alpha synuclein and H&E staining.

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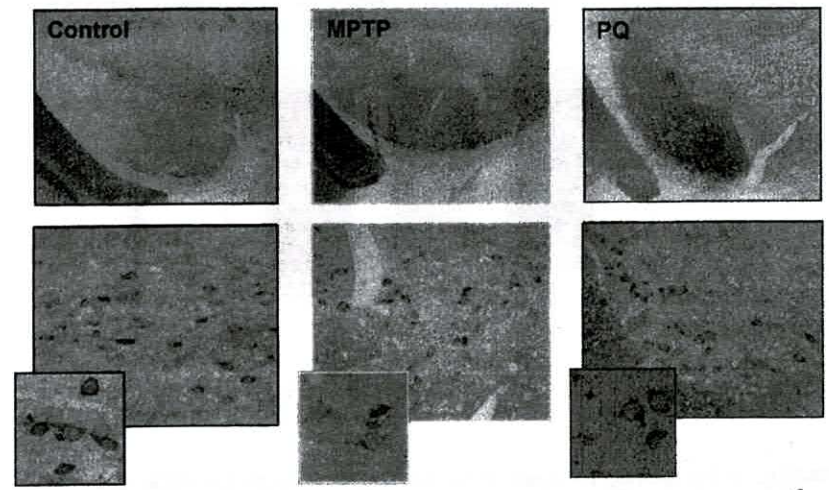
Study 1- Effect of MPTP and Paraquat on α-synuclein expression



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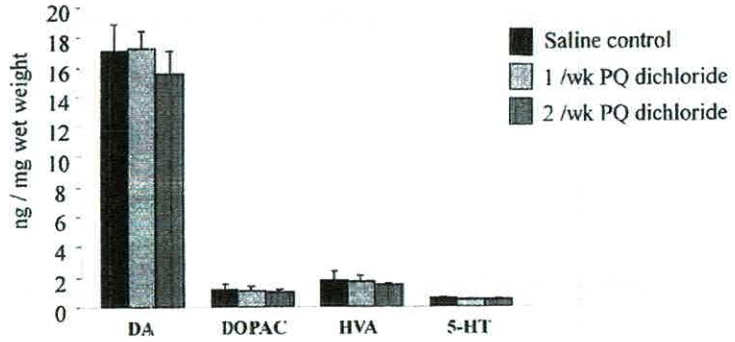
Effects of Dosing Paraquat Twice a Week for 3 Weeks

HPLC analysis of striatal dopamine



Data represents mean ± SD. Data analysed using one way ANOVA followed by Student's t-test. Control . n=8: once a week treated animals received 3 weekly injections of 10 mg/kg PQ dichloride. n=9. twice a week treated animals received 10 mg/kg PQ dichloride twice a week for 3 weeks. n=8.

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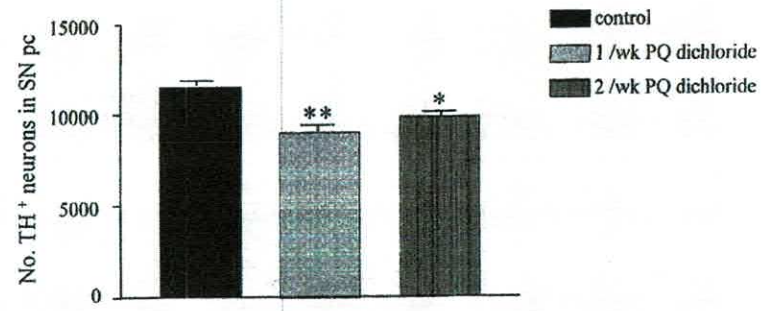
SYNG-PQ-00493416

Study 2- Effects of Dosing Paraquat Twice a Week for 3 Weeks

TH^+ cell counts in SNpc



Data represents mean ± SEM. Saline control group received 3 weekly i.p. injections of Sterile saline. n=8: Treated once a week group received 3 weekly injections of 10 mg/kg PQ dichloride. n=9: Treated twice a week group received injections of 10 mg/kg compound-X twice weekly over 3 weeks. n=9. Values analysed by one way ANOVA followed by Newman-Keuls post test. **p<0.001 *p<0.01

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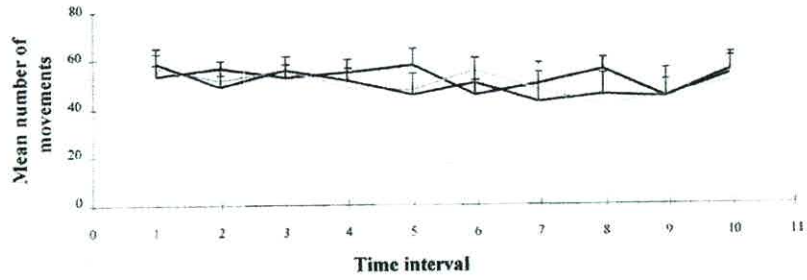
SYNG-PQ-00493414

Study 2- Effects of Dosing Paraquat Twice a Week for 3 Weeks

Locomotor activity

Mean number of total movements **7 days** after final dose



Saline

10 mg/kg PQ dichloride twice per week

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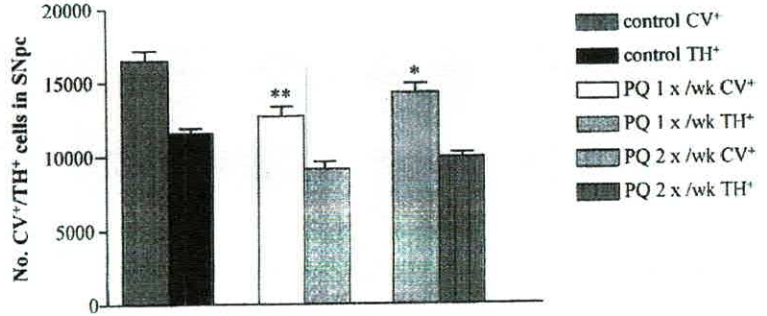
SYNG-PQ-00493417

Study 2- Effects of Dosing PQ 2 x/ week for 3 weeks

CV^+ cell counts in SNpc: 23% ↓ TH+ cells (1x /wk) ; 13% ↓ TH+ cells (2x /wk):



Data represents mean ± SEM. Saline control group received 3 weekly i.p. injections of sterile saline. n=8: PQ 1 x week group received 3 weekly injections of 10mg/kg PQ dichloride. n=9: PQ 2 x week group received injections of 10mg/kg PQ dichloride twice weekly over 3 weeks. n=9. Values analysed by one way ANOVA followed by Newman-Keuls post test. **p<0.001 *p<0.05

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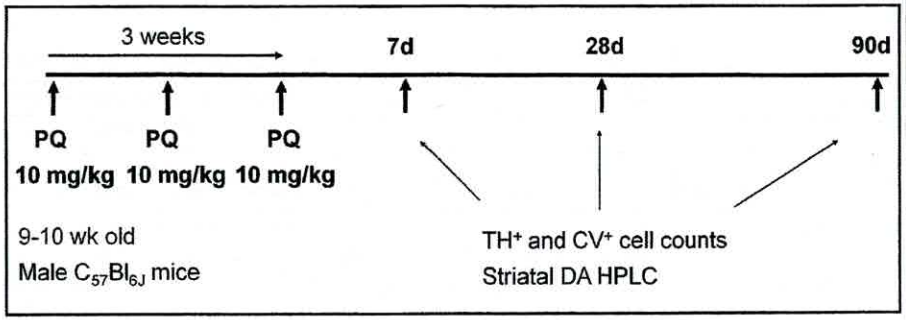
SYNG-PQ-00493415

Study 3- Investigating the Time Course and Reversibility of PQ Induced DA Cell Loss in the C57 Black Mouse



- Same dosing regimen, strain and age of mice as in previous studies
- i.p. injections
- n of 9 per group
- Control groups for each time point receiving 3 injections of sterile physiological saline

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Study 2- Effects of Dosing PQ 2 x/ week for 3 weeks

Conclusion

- Dosing with 10 mg/kg paraquat 1x /week for 3 weeks resulted in a statistically significant reduction in cell number in the SNpc *(21%)*
- Dosing with 10 mg/kg paraquat 2x /week for 3 weeks did not enhance the cell loss
- No statistically significant change in the concentration of striatal DA or DA turnover was observed with 1x /week or 2x /week dosing with PQ
- When dosed once or twice a week for 3 weeks, 10 mg/kg paraquat did not induce any significant changes in locomotor activity

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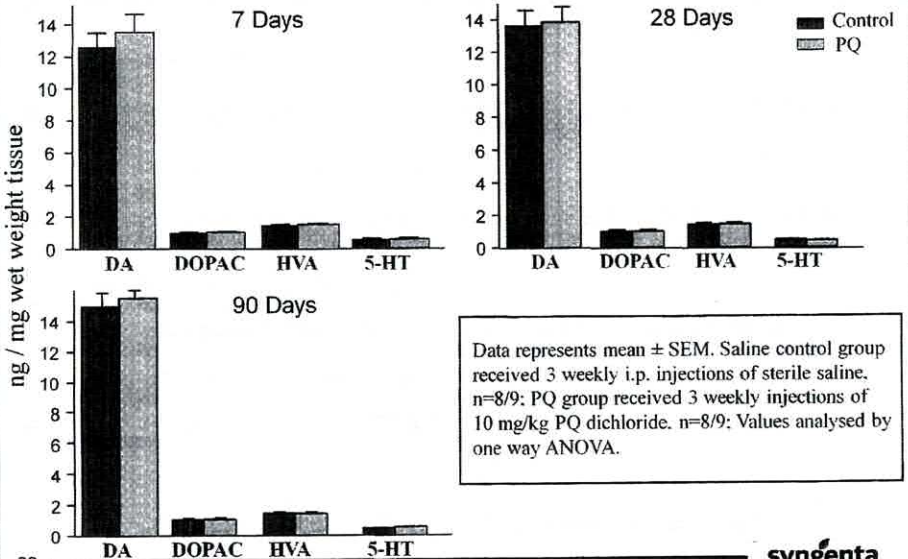
Study 3- Effect of Paraquat on the Concentration of Striatal Dopamine and its Metabolites



Data represents mean ± SEM. Saline control group received 3 weekly i.p. injections of sterile saline. n=8/9; PQ group received 3 weekly injections of 10 mg/kg PQ dichloride. n=8/9; Values analysed by one way ANOVA.

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Effect of Paraquat on Cell Number in the SNpc

- There would appear to be a significant reduction in the number of TH^+ cells in the SNpc at the 7d, 28d and 90d time points. The loss observed at 7d is consistent with the magnitude of loss reported at this time point in previous CTL studies *(XM7371, XM7258)*.
- The reduction in TH^+ cell number is accompanied by a significant reduction in CV^+ cell number at all 3 time points.
- There is no significant change in the number of CV^+ TH^- neurons at any of the 3 time points.
- The percentage reductions are comparable with data from the Di Monte group.

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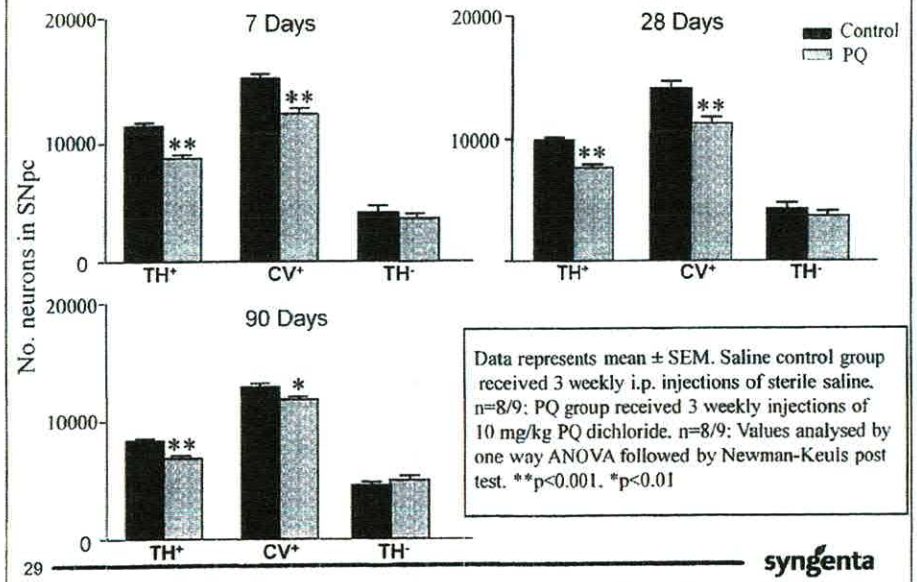
Study 3- Effect of Paraquat on Cell Number in the SNpc



Data represents mean ± SEM. Saline control group received 3 weekly i.p. injections of sterile saline, n=8/9; PQ group received 3 weekly injections of 10 mg/kg PQ dichloride. n=8/9; Values analysed by one way ANOVA followed by Newman-Keuls post test. **p<0.001, *p<0.01

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PQ Dosing Studies at CTL - Conclusions

Endpoint	Reported in the literature?	Reported by the Parkinson's Institute?	Observed in CTL studies?
In vivo			
Loss of striatal DA	Yes (7-10%)	No	No
Significant cell loss in SNpc	**Yes**	**Yes 25-30%**	**Yes 21-24%**
Progressive	Yes (over 3 mths)	No	No (out to 90 days)
Dose dependent	Yes	Yes (1, 5, 10 mg/kg)	-
Increased loss with increased frequency	-	No	No
Clinical / behavioural observations of PQ induced toxicity	Yes	No	No
Increase in markers of oxidative stress	Yes	Yes	-
Increase in markers of inflammation	Yes	Yes	-
Increased alpha synuclein expression	Yes	Yes	Yes
In vitro			
Interaction with DA transporter	No	-	No
Binding at DA receptors	-	-	No

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Study 3- Investigating the Time Course of PQ Effects

Conclusion

- 10 mg/kg paraquat dichloride produced a statistically significant reduction in DA cell number in the SNpc when dosed once a week for 3 weeks to male $C_{57}Bl_{6J}$ mice
- The degree of cell loss observed 7 days after dosing was consistent with our previous studies *(24%)*
- The degree of cell loss was not progressive with no further increase in the magnitude of cell loss at 28 *(24%)* or 90 *(18%)* days
- No statistically significant change in the concentration of striatal DA or DA turnover was observed at 7 , 28 or 90 days after the final dose

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4 Compound Study- Experimental Design

Does dosing with any compound at a high enough dose result in nigrostriatal toxicity?

- Male $C_{57}Bl_{6J}$ mice received 3 weekly i.p. injections of the maximum tolerated dose (MTD) of :
  - **caffeine** (84 mg/kg)
  - **N-ethylmaleimide** (NEM) (2 mg/kg)
  - **antimycin A** (1.3 mg/kg)
  - **paracetamol** (300 mg/kg)
- Mice were observed for up to 4 hours post dosing for clinical signs of toxicity
- Mice were killed 7 days after the final dose and tissue processed for
  - HPLC analysis of striatal dopamine and its key metabolites
  - TH immunohistochemistry and cell counts (stereology)

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Summary and Conclusions

- Paraquat would appear to have the ability to induce nigral cell loss in the mouse without an accompanying reduction in the concentration of striatal dopamine.
- In contrast to some reports in the literature systemic administration of paraquat does not appear to induce a reduction in locomotor activity.
- The ability of paraquat to induce the characteristic pathology and symptoms of PD when administered to rodents has not therefore been conclusively proven across laboratories.
- It should also be remembered that the doses and route of administration used in laboratory studies are not relevant to potential human exposure.
- A role for environmental factors in the aetiology of PD is clearly an area that merits further investigation.

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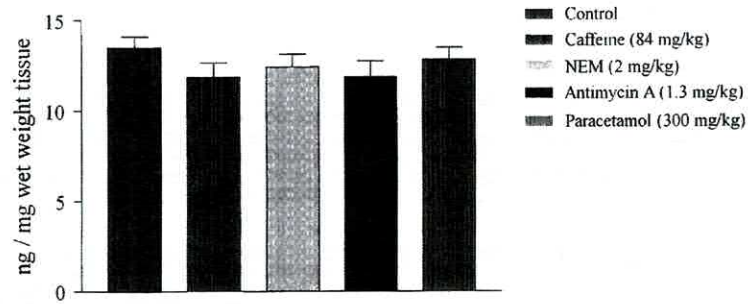
4 Compound Study - Effect on Striatal Dopamine



Data represents mean ± SEM. Data analysed using Student's t-test. Compounds were administered i.p. once a week for 3 weeks at MTD doses and animals terminated 7 days after the last dose. n = 10 per group, control, caffeine, NEM and paracetamol; n = 9 antimycin A.

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4 Compound Study- Conclusions

- Dosing with caffeine, NEM, antimycin A or paracetamol at doses near to or at their maximum tolerated dose did not result in nigrostriatal toxicity in the C57 black mouse.
- The data would suggest that PQ induced cell loss in the SNpc is not likely to be attributable to a 'general' toxicity associated with dosing a compound at high doses rather it suggests a selective effect on vulnerable dopaminergic cells within the SNpc.

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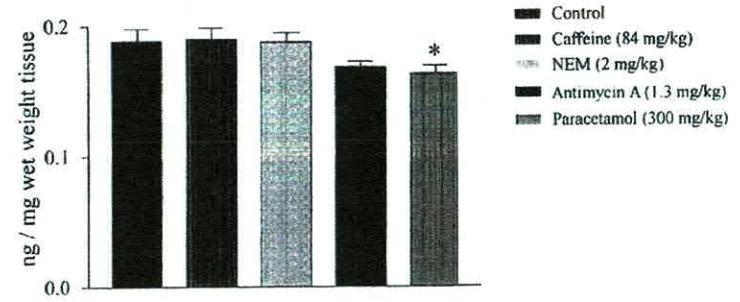
4 Compound Study - Effect on Striatal DA Turnover

Turnover expressed as [DOPAC] + [HVA] / [DA]



Data represents mean ± SEM. Data analysed using Student's t-test. Compounds were administered i.p. once a week for 3 weeks at MTD doses and animals terminated 7 days after the last dose. n = 9 per group. control. caffeine. NEM and paracetamol: n = 8 antimycin A. * p = 0.0489

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Investigating PQ induced nigrostriatal deficits in the rat

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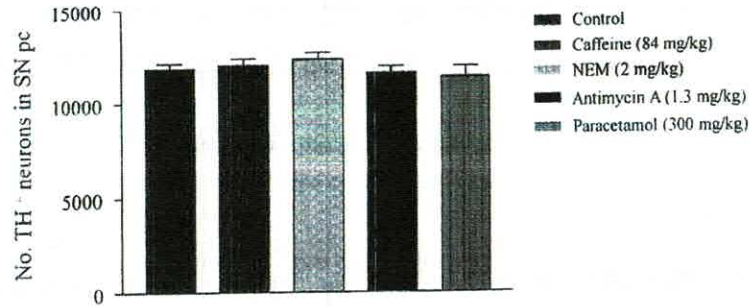
4 Compound Study - TH$^+$ Cell Counts in the SNpc



Data represents mean ± SEM. n=10 per group. Control control group received 3 weekly i.p. injections of sterile saline. Data analysed by one way ANOVA followed by Newman-Keuls post test.

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Investigating PQ induced nigrostriatal deficits in the rat

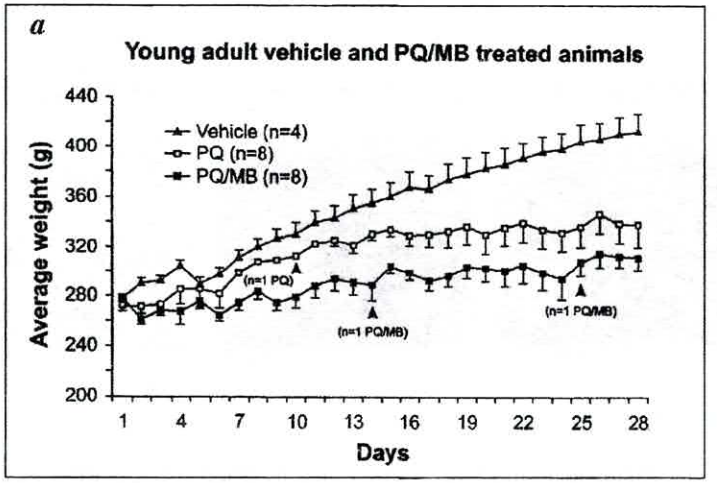
Aim - To investigate reported neuronal cell loss in the rat following systemic administration of PQ dichloride.

Methodology *(Cicchetti et al., 2005)*

- Male 8 week old Sprague Dawley rats
- 10 mg/kg PQ dichloride i.p. twice a week for 4 weeks
- n = 10 per group (1 control and 1 treated group)
- Animals observed for up to 2 hrs post dosing for clinical signs of toxicity
- Body weights recorded daily for the duration of the study
- Animals killed 24 hours after the final dose
- TH cell counts performed in the SNpc using free floating IHC and stereology
- *Striatal tissue was also taken for possible analysis of striatal dopamine concentration at a later date*

Investigating PQ induced nigrostriatal deficits in the rat

Body weight data from Cicchetti *et al.*, 2005



Investigating PQ induced nigrostriatal deficits in the rat

Clinical signs

- The following clinical signs of toxicity were observed after dosing with PQ dichloride in the Sprague Dawley rat:

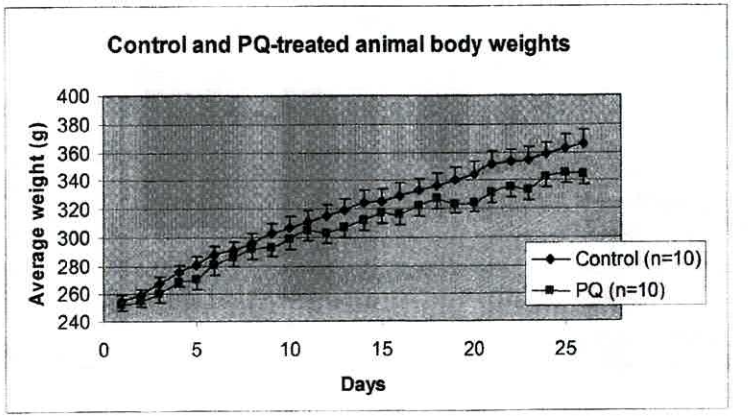
  piloerection, reduced activity, pinched in sides, upward curvature of the spine, altered breathing rate, altered breathing depth, hunched appearance, tip toe gait

- When exhibited, clinical signs were observed for ~1-2 days post dosing with animals recovering and appearing normal prior to receiving the next dose of PQ.

- One PQ treated animal (no. 16) exhibited S-M clinical signs following the first PQ injection (possible miss dosing). The animal also lost weight over several days post dosing. For this reason the animal did not receive the second PQ injection, thus receiving 7/8 PQ doses in total. This animal will be excluded from the data analysis.

Investigating PQ induced nigrostriatal deficits in the rat

Effects on body weight



Weight loss in the PQ group was well within the 25% ↓ acceptable under the licence banding

XR7641 – Distribution of Activated Microglia (from double staining with TH and Ox-42 antibodies)

Control		PQ treated	
Animal no.		Animal no.	
1	++	1	0
2	+	2	++
3	0	3	0
4	+	4	0
5	++	5	0

Key
0 = No activated cells
+ = Few (1-10) within SNpc
++ = Few (1-10) within SNpc and SNR
+++ = Significant (11-20) within SNpc

PQ treated animals received 10 mg/kg PQ dichloride i.p. twice a week for 3 weeks (n=5). Controls received injections of sterile physiological saline (n=5). Animals were killed 24 hours after the final injection.

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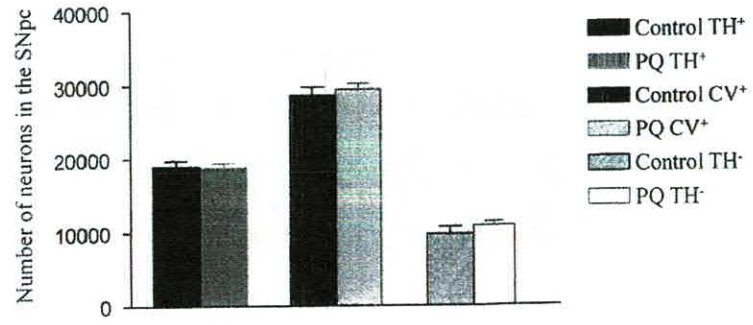
XR7641 - Effect of PQ on Cell Number in the Rat SNpc



Data represents mean ± SEM. Data analysed using one way ANOVA followed by Newman–Keuls post test. PQ treated animals received 10 mg/kg PQ dichloride i.p. twice a week for 3 weeks (n=8/9). Controls received injections of sterile physiological saline (n=9/10). Animals were killed 24 hours after the final injection.

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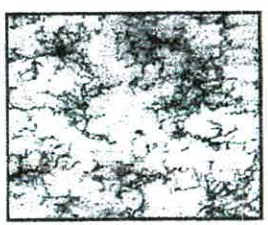
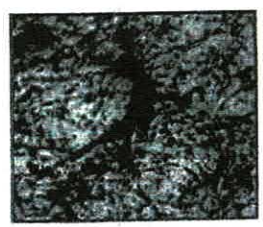
TH and OX-42 double label in the SNpc of the Rat



Typical Morphology of Resting Microglia

Activated Microglial Cell



Activated Microglial Cell and DA Neuron

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XR7641 – Effect of PQ on Cell Number in the Rat SNpc

TH+		CV+		TH-	
Control	PQ	Control	PQ	Control	PQ
21323	19497	31386	29118	10063	8854
19637	20264	31539	29110	11902	9365
16750	19745	26762	34030	10012	13302
18922	20728	29013	31030	10091	11237
21986	19793	34048	27819	12062	10609
19013	17210	27832	26853	7772	11878
20060	14975	28774	28181	14118	10003
14656	18178	26029	29318	5563	11171
20466	18147	22474		5898	
16576					
Mean	18939	18726	28651	29432	9720	10802
SD	2304	1808	3434	2227	2864	1428
±SEM	728.5	602.6	1145	787.5	954.6	504.8

Data analysed using one way ANOVA followed by Newman-Keuls post test. PQ treated animals received 10 mg/kg PQ dichloride i.p. twice a week for 3 weeks (n=8/9). Controls received injections of sterile physiological saline (n=9/10). Animals were killed 24 hours after the final injection.

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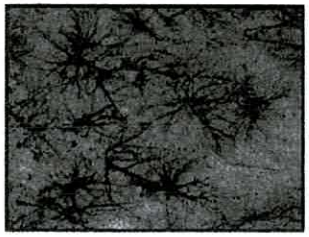
TH and GFAP double label in the SNpc of the Rat



Typical Astrocyte Morphology

Resting Astrocyte Within the SNpc

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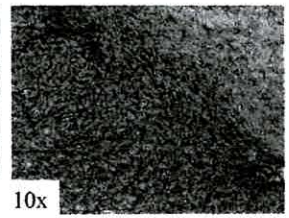
TH and OX-42 double label in the SNpc of the Rat

Control animal

5x

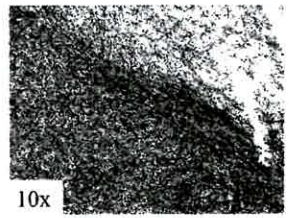


10x

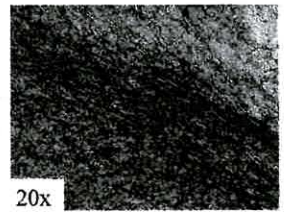
20x

PQ treated animal

5x



10x



20x

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XR7641 – Distribution of activated astrocytes (from double staining with TH and GFAP antibodies)

Control		PQ treated	
Animal no.		Animal no.	
1	0	1	0
2	0	2	0
3	0	3	0
4	0	4	0
5	0	5	0

Key

0 = No activated cells

+ = Few (1-10) within SNpc

++ = Few (1-10) within SNpc and SNR

+++ = Significant (11-20) within SNpc

PQ treated animals received 10 mg/kg PQ dichloride i.p. twice a week for 3 weeks (n=5). Controls received injections of sterile physiological saline (n=5). Animals were killed 24 hours after the final injection.

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