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ADEQUACY AND CONSISTENCY OF ANIMAL STUDIES TO EVALUATE THE NEUROTOXICITY OF CHRONIC LOW LEVEL MANGANESE EXPOSURE IN HUMANS

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Running title: Adequacy of Animal Studies to Evaluate Mn Toxicity

ABSTRACT

The adequacy of existing animal studies to understand the effects of chronic low-level manganese exposures in humans is unclear. Here, a collection of sub-chronic to chronic rodent and non-human primate studies was evaluated to determine whether there is a consistent dose – response relationship among studies, whether there is a progression of effects with increasing dose, and whether these studies are adequate for evaluating the neurotoxicity of chronic low-level manganese exposures in humans. Neurochemical and behavioral effects were compared along the axis of estimated internal cumulative manganese dose, independent of the route of exposure. In rodents, motor effects emerged at cumulative doses below those where occupationally exposed humans start to show motor deficits. The main neurochemical effects in rodents were an increase in striatal GABA concentration throughout the internal cumulative dose range of 18 to 5300 mg Mn/Kg but a variable effect on striatal dopamine concentration emerging at internal cumulative doses above ~200 mg Mn/Kg. Monkey studies showed motor deficits and effects on the globus pallidus at relatively low doses and consistent harmful effects on both the globus pallidus and the caudate and putamen at higher doses (> 260 mg Mn/Kg). Internal cumulative manganese doses of animal studies extend more than two orders of magnitude (<1 to 5300 mg Mn/Kg) above the doses at which occupationally exposed humans show neurological dysfunction (10-15 mg Mn/Kg). Since the animal data indicate that manganese neurotoxicity may be different at low compared to elevated exposures, most existing animal model studies might be of

limited relevance for the risk assessment of chronic low-level manganese exposure to humans.

INTRODUCTION

Manganese is a micronutrient essential for a diverse range of biological functions but deleterious to the central nervous system at elevated exposures. The Parkinsonian symptoms that characterize the disorder of manganism were first observed by Couper in workers of the chemical industry more than 150 year ago (Couper 1837). Since then, neurotoxic effects have been reported in workers from a variety of occupations at risk for elevated exposures, such as miners, welders, and battery and ferroalloy industries workers (Rodier 1955; Chandra et al 1981; Huang et al 1989, 1993; Roels et al 1992; Racette et al 2005a). While the existence of neurological risks associated with occupational exposures is well established, potential long term human health consequences from environmental low-level chronic manganese exposure are not known. There is a pressing need to address this concern because of the expected increase in environmental manganese levels due to the utilization of the gasoline additive methylcyclopentadienyl manganese tricarbonyl (MMT) in the US and in a number of other countries (Davis et al 1998; Frumkin and Solomon 1997, Lyznicki et al 1999; Thibault et al 2002; Zayed 2001; Blumberg and Walsh 2004; Rollin et al 2005). Combustion of MMT containing gasoline generates tailpipe emissions of manganese phosphates, sulfates and oxides (Molders et al 2001; Ressler et al 1999). More than 99% of the particles emitted in MMT combustion are in the respirable fraction (<5 μM), and more than 85% of them are less than 1 μM (Ardeleanu et al 1999).

Available evidence indicating the existence of a continuum of dysfunction under a wide range of human exposures suggests that long-term environmental exposure to manganese could pose a significant risk to human health (Mergler 1999; Mergler and Baldwin 1997). This continuum ranges from severe motor dysfunction leading to overt signs of manganism in specific work environments (dust manganese content > 1 mg/m^3), to sub-clinical motor, memory and emotional effects at low exposure levels in occupational settings (dust manganese content 0.2-1 mg/m^3; Iregren 1999), to subtle (non-clinical) behavioral, emotional and motor effects under lifetime environmental exposures close to those deemed safe (total particulate Mn content ~0.05 μg/m^3). For example, exposure to manganese in occupational settings induced sub-clinical neurological signs consistent with early manganism (Chandra et al 1981; Tanaka and Lieben 1969), and early non-clinical neurofunctional deficits in visual reaction time, eye-hand coordination, and hand steadiness and memory, as well as behavioral effects (lower activity levels, sexual drive, and olfactory perception; fatigue and irritability) (Roels et al 1987, 1992; Iregren 1990; Kim et al 2005; Lucchini et al 1995, 1999; Mergler et al 1994). High prevalence of Parkinsonian disturbances was also observed among residents in the vicinities of ferro-manganese plants (Lucchini et al 2003). Exposure to manganese in groundwater at levels of 0.082 to 0.25 mg/L was linked to an increased prevalence of neurological signs suggestive of extrapyramidal dysfunction in an elderly population (Kondakis et al 1989). Finally, exposure to levels of airborne manganese close to the US EPA reference concentration (RfC) of 0.05 μg/m^3

was associated with early symptoms of motor deficits and mood disorders in residents of Southwest Québec, similar to those seen in occupationally exposed workers (Mergler et al 1999; Baldwin et al 1999; Beuter et al 1999; Bowler et al 1999; Hudnell 1999).

Prompted in part by the concern over increased chronic environmental manganese exposure from MMT combustion in gasoline, regulatory agencies performed risk assessment analyses and estimated safe levels of inhaled manganese for the general population (Davis et al 1998; Egyed and Wood 1996; Wood and Egyed 1994). The calculation of protective levels was based on an extrapolation of the cumulative inhaled manganese dose of occupationally exposed workers with early signs of neurobehavioral adverse effects to a lifetime exposure in the general population. The risk assessment also included adjustments intended to (1) protect sensitive sub-populations, (2) account for the use of a LOAEL (lowest observed adverse effect level) instead of a NOAEL (no observed adverse effect level), and (3) account for the limited nature of the database that the risk extrapolation is based upon (Davis et al 1998; Egyed and Wood 1996). Key assumptions in the extrapolation approach are that effects produced at high and low doses differ only in their severity and not in the underlying toxic mechanism, and effects (i.e., symptoms) observed at high exposures over relatively short durations would occur also at lower exposures spread over longer periods of time. The latter assumption is partly substantiated by occupational studies showing that neurotoxicity in workers exposed to

airborne manganese over different lengths of time was related to the cumulative amount of manganese inhaled rather than to the manganese concentration in air (Lucchini et al 1995, 1999; Roels et al 1992). However, currently there is insufficient evidence from non-occupational studies of prolonged exposure to validate the extrapolation of the dose–response relationship obtained in occupational settings to lower chronic environmental exposures. Moreover, the suite of toxicity outcomes assessed in occupational studies may not represent the entire range of deleterious effects that may be elicited after a lifetime exposure to lower manganese levels.

These limitations may, in principle, be addressed through studies in model vertebrate species, in which a wide range of exposure doses and durations, as well as physiologic, molecular, and neurochemical outcome measures can be readily evaluated. In particular, animal studies (1) are essential for the elucidation of mechanisms of manganese toxicity observed in humans; (2) offer the possibility to test for compensated cellular-based effects that do not have a functional (i.e., behavioral) manifestation, and thus may go undetected in human occupational studies; (3) enable joint evaluation of cellular and functional outcomes over a range of doses, leading to the identification of thresholds where cellular protective and compensatory mechanisms are overwhelmed and behavioral dysfunction emerges; and, (4) provide a model to evaluate possible therapeutic treatments for early effects of manganese exposure in humans. As such, animal studies aid the risk characterization process and can be

instrumental in validating the assumptions implicit in the risk assessment for manganese. For example, animal studies may be invaluable in testing the assumption that the critical effects associated with a LOAEL used as the basis for the RfC calculation is indeed the first adverse effect or whether there are other effects at lower doses, not observed or tested in humans occupationally exposed, that should serve as a more sensitive indicator of manganese toxicity.

Numerous animal studies on manganese neurotoxicity have been conducted over the last 30 years, mostly in rodents. The majority of these studies appear to have been designed to understand the mechanism(s) underlying manganese toxicity seen in occupationally exposed subjects, and thus have utilized very elevated manganese dosing regimens and focused on a relatively limited number of outcome measures. In addition, these studies very rarely utilized more than one dose. Thus, the extent to which these animal studies may be of utility to understand the risk for adverse effects in humans suffering chronic low-level environmental exposures is not clear. Some recent animal studies have started to address the potential effects of low-level environmental exposures by administering environmentally relevant doses via exposure routes important in humans, though these studies are relatively few in number (Dorman et al 2005; Normandin et al 2002, 2004; Salehi et al 2003; Tapin et al 2005).

Here multiple animal studies on manganese neurotoxicity were critically analyzed to address the following questions: (1) is a consistent dose-response relationship observed across animal studies? (2) is there evidence for a sequence of toxic responses with increasing manganese dose? and, (3) is the

dosimetry data derived from the animal studies sufficient to inform the human risk assessment of environmental manganese exposures? Answering these questions may assist in the identification of existing gaps in the current data and in the design of future animal studies to better aid the risk assessment of chronic low-level manganese exposure in humans.

METHODS

1. *Selection of studies*: Over 100 hundred papers on manganese neurotoxocity in rodents and monkeys were identified from searches of PUBMED, BIOSIS, AND TOXNET databases, and from published compilations of studies of manganese toxicity (e.g.,ATSDR 2000). From this large group, rodent studies were selected for analyses according to the following criteria: (1) the manganese route of exposure was either inhalation, intraperitoneal (i.p.), food, water, gavage or tail vein; studies that used the intracranial route were not included; (2) manganese was administered as an inorganic species; (3) treatment duration was sub-chronic to chronic (longer than 30 days); (4) animals were age 21 days or older (i.e., post-weaning); (5) reported outcomes were neurochemical measures in the basal ganglia and/or indices of motor dysfunction. Studies that reported only manganese brain levels were not included; and (6) outcomes were reported in more than one study. Thirty rodent papers met these criteria. All but three of these studies (Gwiazda et al 2002; Witholt et al 2000; Kosicka et al 1983) utilized male rodents. A total of 16 non-human primate studies were found and all were included in the analysis because

of the greater relevance of non-human primates to human risk characterization. Six monkey studies utilized males, 3 used females, 2 used both sexes and 5 did not report gender. In order to be as inclusive as possible, there was no attempt to select or exclude studies based on quality, i.e., outcomes are reported here as described by the authors.

2. Dose estimate: Estimated internal cumulative doses (ICD_{Mn}) were calculated for all outcomes reported in the studies. The estimated internal cumulative dose is the total amount of manganese that was taken-up into the circulatory system by the time the endpoint was detected. For behavioral outcomes the ICD_{Mn} was calculated for the period from beginning of treatment to the first time the outcome was observed. For neurochemical or histological endpoints, which were measured after animals were sacrificed, the ICD_{Mn} was calculated from beginning to end of treatment. The estimated internal cumulative manganese doses in units of mg Mn/Kg body weight were calculated according to the formula:

$$ICD_{Mn} = \frac{\text{Dose} * f_{Mn} * C * T * abs}{B.W.} \qquad (1),$$

where Dose is the nominal dose (e.g., mg of $MnCl_2.4H_20$ /mL water, mg Mn/m^3, etc.), f_{Mn} is the weight % of manganese in the compound administered (e.g., 0.28 or 28% in $MnCl_2.4H_20$), C is the measured or estimated daily consumption of the

exposure media (water, food, air), T is treatment duration, *abs* is the estimated fraction of manganese absorbed into the blood stream, and B.W. is body weight.

3. *Studies Comparison*: The severity and sequence of effects with increasing manganese dose were evaluated by plotting the effects reported in each study (i.e., decrease, no effect, or increase relative to control) versus their associated estimated internal cumulative manganese doses (Figures 1 and 2).

Parameter values: Rodent studies

For studies where the route of administration was food, water or gavage a gastrointestinal absorption of 3% (i.e., *abs* = 0.03) was applied. Studies have shown that as manganese intake increases, fractional gastrointestinal absorption decreases in rodents (20 to 0.5%) (Weigand et al 1986; Arnich et al 2004). This coefficient is probably different for each study reviewed here and the choice of 3%, while somewhat arbitrary, is in the range of fractional absorption measured at high manganese intake levels. For i.p. and tail vein routes 100% manganese uptake was assumed (*abs* =1). Unless reported, food and water consumption rates were assumed to be 10% of body weight per day (Sharp and LaRegina 1998) (i.e., C/B.W. = 0.1 in equation (1)).

For inhalation exposures, inhalation rates of 1L/min/Kg body weight for rats and 45 mL/min/Kg for a typical mouse were used (Kennedy and Valentine 1994). Scaling inhaled manganese dose to internal cumulative manganese dose is fraught with uncertainties because particle penetration in the lung, and the corresponding effective dose of manganese absorbed, is determined by the

particle size distribution (Kennedy and Valentine 1994). In contrast to almost all non-inhalation rodent studies, which used the highly water soluble $Mn(II)Cl_2$ salt, inhalation studies utilized manganese compounds with different solubilities (and oxidation states): manganese sulfate ($MnSO_4$), manganese phosphate as the hureaulite mineral $Mn_5(PO_4)_2(PO_3OH)_2.4H_2O$, MnO_2, and elemental manganese. Given these complexities, the most conservative approach was to assume that all inhaled manganese was absorbed (*abs* = 1). Under this assumption, the internal cumulative manganese dose of inhalation studies calculated here most likely overestimates the actual dose absorbed.

Parameter values: Non-human primate studies

The number of non-human primates utilized per treatment in the reviewed studies was small (5 or less), such that specific endpoints are mostly reported for individual animals and not as a group-average outcome of the manganese exposure treatment. Only 6 papers reported group-average neurochemical endpoints of manganese treatment (Ulrich et al 1979a, 1979b, 1979c; Bird et al 1984; Eriksson et al 1992a; Neff et al 1969). Evaluation of motor effects was mostly qualitative with only two studies reporting quantitative tests to evaluate motor response (Eriksson et al 1992a; Newland and Weiss 1992).

The manganese exposure routes used in these non-human primate studies were diverse, encompassing subcutaneous, intravenous, intramuscular, intraperitoneal, inhalation and oral routes. Here, uptakes of 3% for oral exposures (i.e, *abs* = 0.03) and 100% for the other exposure routes (*abs* = 1)

were assumed. While manganese treatment durations often exceeded one month, exposures often consisted of only a few injections of manganese at high concentrations, as opposed to more frequent treatment of repeated injections at lower concentrations, or continuous exposure through water or food more commonly used in the rodent studies. Internal cumulative manganese doses in inhalation studies were calculated assuming complete absorption (*abs* =1, as with rodent inhalation studies), and inhalation rates of 490 mL/min/Kg body weight for squirrel monkeys (Ulrich et al 1977) and 420 mL/min/Kg for rhesus monkeys (Bourne 1975). Published reference body weights of the particular monkey species and age were used when weights were not reported (Mella 1924; Neff et al 1969; Pentschew et al 1963).

The manganese species utilized for manganese exposure in non-human primate studies was Mn(II), Mn(IV) or MMT combustion products having Mn(II), Mn(IV) and a lesser contribution of Mn(III) in Mn_3O_4. Administration of highly water soluble $Mn(II)Cl_2$ is reported in 6 papers, poorly soluble MnO_2 in 9 and MMT combustion products in 1.

RESULTS

Rodents

The range of internal cumulative doses used in rodent studies is wide, spanning more than four orders of magnitude, from 0.26 to 5220 mg Mn/Kg (Figure 1). The studies examined functional outcomes (motor activity), neurochemical changes (striatal monoamine oxidase activity, striatal dopamine,

striatal GABA levels), and histological changes at the cellular level (globus pallidus cell number and striatum cell number).

Manganese effects on motor activity in rodents start to appear at cumulative exposures similar to or even lower than those that start to produce neurobehavioral abnormalities in humans (i.e., 10 to 15 mg Mn/Kg), based on data from Lucchini et al. (1995) and from Roels et al. (1992), as discussed in Wood and Egyed (1994). However, the direction of altered spontaneous motor activity (increase or decrease) varies inconsistently across the entire range of cumulative manganese dose, with no clear relationship emerging between cumulative dose and effect on spontaneous activity.

Among neurochemical outcomes, an enhancing effect of manganese treatment on striatal GABA levels is the most consistent result. This effect was observed across a wide range of internal cumulative doses, from 18 to 5300 mg Mn/Kg. Furthermore, the effect of manganese on striatal GABA emerges at a lower internal cumulative dose than doses where striatal dopamine levels are affected. Overall, no consistent depressing effect of manganese on striatal dopamine is observed. At relatively low internal cumulative doses of manganese (< ~200 mg Mn/Kg), there is either a lack of effect or an enhancing effect of manganese on striatal dopamine concentrations. Only the studies of Chandra and colleagues (Chandra and Shukla 1981; Chandra 1983; Shukla and Chandra 1981) show an enhancing effect of manganese exposure on dopamine, not reproduced by others at cumulative manganese doses below 200 mg Mn/Kg. Notably, the brain manganese levels reported by Chandra and Shukla (1981) are

the highest reported in the reviewed rodent studies. Brain manganese levels of both exposed and control animals (6.21 μg/g wet weight and 2.95 μg/g wet weight, respectively) exceed the brain manganese concentrations reported in any other rodent study, the closest being 2.64 μg/g wet weight for manganese exposed rodents reported by Subhash and Padmashree (1990). At moderate manganese doses (e.g., between ~200 mg Mn/Kg and 2300 mg Mn/Kg), no effect as well as adverse effects (increase or decrease) of manganese on striatal dopamine were observed. At high doses (>2300 mg Mn/Kg), decreases in striatal dopamine were reported.

Results from single studies that evaluated both GABAergic and dopaminergic outcomes are consistent with the existence of a sequence of neurotoxic effects with increasing cumulative dose; starting with GABAergic effects at low internal cumulative doses and progressing to GABAergic and dopaminergic effects at higher internal cumulative doses. For example, at low cumulative doses (72 mg Mn/Kg) Witholt et al. (2000) and Gwiazda et al. (2002) detected manganese effects on striatal GABA concentrations and motor activity , but not on striatal dopamine. Similarly, Tapin et al (2005) reported a reduced number of globus pallidus neuronal cells but no change in striatal cell number at low manganese exposures in the range of cumulative doses that induce behavioral abnormalities in humans. Likewise, no changes in striatal cell numbers were found by Calabresi et al. (2001) and Normandin et al. (2002) over the cumulative dose range ~0.3 to 2000 mg Mn/Kg. At relatively high cumulative manganese doses (5300 mg Mn/Kg) Gianutsos and Murray (1982) detected

effects of manganese on both striatal GABA (increased) and dopamine (decreased) concentrations.

The relationship between manganese cumulative dose and the dopamine degrading enzyme monoamine oxidase (MAO) activity is inconsistent. When an effect is present, it is an increase in MAO activity. However, this effect is not necessarily accompanied by a decrease of striatal dopamine concentration, as one might expect. For example, Chandra and Shukla (1981) reported increases in both MAO activity and striatal dopamine concentration at low cumulative doses (13-107 mg Mn/Kg), while Autissier et al (1982) report a decrease in striatal dopamine with no measurable change in striatal MAO activity at a higher cumulative dose of 267 mg Mn/Kg.

Non-human primates

Overall, the range of manganese doses administered in non-human primate studies extends over several orders of magnitude, from doses where occupationally exposed humans start to show neurological effects (10-15 mg Mn/Kg) to values two orders of magnitude higher (Figure 2). Notably, the cumulative manganese doses at which monkeys start to show motor effects overlap with the cumulative doses at which occupationally exposed humans also start to show signs of neurobehavioral dysfunction. Due to the limited number of non-human primate studies of manganese neurotoxicity and the fact that results are mostly reported for individual animals, attempts to draw general observations

from this dataset are necessarily tentative. The most consistent outcomes produced by manganese exposure are a decrease in striatal dopamine and a deleterious effect on globus pallidus integrity, variously characterized as damaged globus pallidus (Mella 1924), loss of pallidal neurons (Eriksson et al 1987b; Pentschew et al 1963), gliosis (Shinotoh et al 1995), proliferation of Alzhemimer Type II astrocytes (Pentschew et al 1963), and decreased globus pallidus dopamine content (Bird et al 1984).

The cumulative manganese dose at which adverse effects were detected in monkeys also appears highly dependent on the chemical species of manganese administered. Effects due to MnO_2 exposure were mostly elicited at cumulative doses higher than 260 mg Mn/Kg, which coincidentally was the maximum cumulative dose given as $MnCl_2$. This phenomenon may reflect manganese species solubility, as discussed later.

As noted above for the rodent studies, results from single non-human primate studies that evaluated more than one endpoint indicate a sequence of neurotoxic effects of manganese. This progression consists of motor deficits and effects on the globus pallidus at relatively low cumulative doses (between 20 - 70 mg Mn/Kg), and consistent effects on both the globus pallidus and the dopamine-rich caudate and putamen (striatum) at higher doses (> 260 mg Mn/Kg). This is evidenced by observations in several studies. Newland (1999) reported motor disturbances at low cumulative doses (10 - 30 mg Mn/Kg) and elevated magnetic resonance imaging-measured manganese accumulation in the globus pallidus and substantia nigra, but not in the caudate and putamen. Increases in

manganese levels in the caudate and putamen were evident only at higher cumulative doses. Shinotoh et al. (1995) and Olanow et al. (1996) also reported manganese effects on motor performance at low cumulative doses (20 - 40 mg Mn/Kg), and observed gliosis in the globus pallidus but no effect on the nigrostriatal pathway at the quantitatively higher cumulative doses measured at the end of treatment (70 - 80 mg Mn/Kg). Finally, motor disturbances were associated with degeneration of the globus pallidus, but not the striatum at a relatively high internal cumulative dose of 632 mg Mn/Kg given as MnO_2 (Pentschew et al 1963), while at the much higher cumulative dose of 2000 mg Mn/Kg (as MnO_2) motor deficits were accompanied by neurochemical effects on both the globus pallidus and striatum (Eriksson et al 1987b).

DISCUSSION

The first aim of this critical analysis is to evaluate whether a consistent dose-response relationship can be inferred from a joint evaluation of all sub-chronic and chronic animals studies. A relatively consistent picture that emerges is that manganese exposure, regardless of route, chemical species given, or to some extent the cumulative dose administered produces enhancement of striatal GABA levels, disturbances of the GABA-rich globus pallidus, and disturbances in motor function (Figure 1, 2). In rodents, effects on motor function appear at doses between 1 to 10 mg Mn/Kg. These doses are below those where occupationally exposed workers start to show motor deficits (10-15 mg Mn/Kg). Manganese effects on neurochemical measures were evaluated only at doses

above 10 mg Mn/Kg, thus, it is not possible to assess from the rodent studies reviewed here the relationship between the appearance of a motor/behavioral effect with the appearance of a neurochemical effect. The limited evidence suggests that motor effects appear before cellular loss in the globus pallidus. In non-human primates motor deficits are also observed at low internal cumulative doses (below 10 mg Mn/Kg) and without effects on globus pallidus cell integrity.

The appearance of manganese neurotoxicity is in part determined by the chemical species administered, probably due to their different solubilities. Analysis of the non-human primate studies shows that the water soluble $MnCl_2$ elicits toxic outcomes at lower cumulative doses than the relatively insoluble MnO_2, independent of route of exposure. In agreement with this, manganese sulfate, a combustion product of MMT, has been shown to be more readily cleared from the lung and to produce higher striatal manganese levels following inhalation exposures than other less soluble MMT combustion products, such as manganese phosphate (hureaulite) and the manganese oxide hausmannite (Mn_3O_4) (Dorman et al 2001). Furthermore, a decrease in motor activity was observed in rodents after inhalation exposure to a manganese sulfate/phosphate mixture but not after exposure to an equivalent amount of manganese phosphate only (Normandin et al 2004). These data suggest that the current Reference Concentration (RfC) or safe level of inhaled manganese for the general population (Davis et al 1998; Egyed and Wood 1996; Wood and Egyed 1994) may not be sufficiently protective because the RfC estimates are based on data

from an occupational study (Roels et al 1992) where workers were exposed to the relatively insoluble MnO_2 (Weast 1985).

The second aim of this analysis is to determine whether there is a progression of neurotoxic effects with increasing manganese dose. Indeed, manganese appears to affect the globus pallidus and produce an enhancing effect on striatal GABA levels over a wide range of cumulative doses, but the depressing effect of manganese on striatal dopamine is only seen at very high cumulative doses. However, the threshold cumulative manganese dose where a dopamine effect emerges, and the directional trend of this initial manifestation, are not clear. In rodents, a manganese effect on dopamine appears at doses of 200 mg Mn/Kg and in monkeys at doses higher than 250 mg Mn/Kg (this latter value may be conservatively high due to the low solubility of MnO_2 utilized in some non-human primate studies) (Figure 1, 2). The observation of a progression of effects with increasing cumulative dose casts doubt into the validity of the risk assessment approach of extrapolating dosimetry data from human occupational studies to lifetime environmental exposures; because this calculation assumes that effects produced at high and low exposures differ only in their severity and not in the underlying toxic mechanism.

It has become accepted that the hallmark neurochemical outcome of manganese neurotixicity is a decrease in striatal dopamine (Cotzias et al., 1976), on the basis of the similarities in motor symptoms between manganism and

Parkinson's disease, and the fact that one of the main features of Parkinson's disease is striatal dopamine depletion. However, the data summarized here suggests that manganese depletes dopamine only at elevated exposures. This raises the intriguing possibility that manganese exposure may both induce atypical Parkinsonism (based on pallidal effects) and contribute to more typical Parkinsonism (based on dopaminergic effects). While a distinction has been made between the motor effects induced by manganese exposure and those observed in Parkinson's disease (Calne et al 1994), a few recent studies propose a higher incidence of Parkinson's disease in workers occupationally exposed to manganese (Gorell et al 1999; Racette et al 2005b). Manganese exposure could be a risk factor of Parkinson's disease if manganese were to accelerate depletion of striatal dopamine after sustained occupational exposure, and precipitate the appearance of Parkinson's disease like-symptoms.

The emergence of a manganese effect on the striatal dopamine system at high cumulative doses could explain the mixed outcomes of studies that evaluated L-DOPA treatment on manganese-induced dysfunction. Based on the evidence from the studies reviewed here, it is expected that L-DOPA would be effective only at the highest cumulative manganese exposures where dopamine depletion occurs, but not at lower exposures before dopamine depletion manifests. Several studies have reported that L-DOPA treatment was effective in cases of manganese poisoning (Huang et al 1989; Mena et al 1970; Rosenstock et al 1971), while other (double-blind) studies have reported that L-DOPA therapy

was ineffective in the treatment of motor dysfunction associated with high manganese exposure (Koller et al 2004; Lu et al 1994). These observations are consistent with the proposition that dopamine depletion emerges as the ultimate effect on the dopaminergic system, though this effect may be preceded by changes in other parameters, such as dopamine receptor affinity and density (Shinotoh et al 1997), dopamine reuptake ability (Kim et al 2005; Racette et al 2005b), dopamine production or dopamine vesicular storage.

A third objective of this review is to evaluate the utility of dosimetry data from animal studies to inform the risk assessment. Data presented here show that the vast majority of animal studies conducted to date have used cumulative exposure regimens that exceed the exposure levels experienced by occupationally exposed humans by two orders of magnitude or more, and thus may be inadequate to evaluate the risk of chronic environmental manganese exposures in humans. The existence of different kinds of toxic effects at low versus high cumulative doses suggests that only animal studies with low cumulative doses should be considered for estimating safe levels of exposure to humans. High cumulative doses are not scalable to lower levels of exposure because they elicit different kinds of effects, suggesting different underlying mechanisms of toxicity. Further, because inhalation studies in rodents show toxic effects at cumulative doses even lower than those where occupationally exposed humans start to show adverse effects (Salehi et al., 2003; Tapin et al., 2005, Figure 1) the most adequate animal studies to address lifetime low level

exposure in humans should administer a range of cumulative doses lower that those measured in occupational settings.

To facilitate comparison across studies of the severity and sequence of effects with increasing dose, the body weight normalized internal cumulative manganese dose was used here as the measure of exposure, independent of the route of administration. This index of exposure is preferred over the amount of manganese administered per dose (nominal dose), which does not take into consideration the duration of exposure. The use of cumulative dose as a relevant metric of exposure producing toxic effects is supported by the observed relationship in occupational settings between inhaled cumulative manganese dose, calculated for each worker by multiplying average airborne manganese concentrations by the length of exposure, and the severity of motor deficits (Lucchini et al 1995; Roels et al 1992). By considering the internalized cumulative dose, studies with different routes of administration are normalized to a common axis because the amount of manganese given over the different routes is converted to the manganese load delivered to the circulatory system, independent of the path of exposure. The possible effects of exposure route on the toxic outcomes were not considered here, because it was assumed that the same circulatory load of manganese produced the same target site dose of manganese, regardless of the route of exposure. The default assumption in risk assessment is to assume that the target site dose is the ultimate determinant of effect, and chemicals that produce target site toxicity by one route of exposure

will do so by any other route of exposure, as long as the target site doses are comparable (EPA 2004).

The precise location along the axis of internal cumulative dose of outcomes from inhalation studies relative to those from other studies is uncertain. With the utilization of internalized cumulative dose, all studies were normalized according to the systemic manganese load, and ultimately to the target size dose. However, in the case of inhalation, the relationship between cumulative dose and target size dose may be different from that of other exposure routes, as it appears that a fraction of the manganese inhaled may bypass the circulatory system and be directly taken into the brain through retrograde transport via the olfactory neuronal pathway and the trigeminal system (Brenneman et al 2000; Dorman et al 2002, 2004; Henriksson and Tjalve 2000, Lewis et al 2005). Thus, manganese uptake via inhalation may produce higher target (brain) doses than equal manganese uptake via other routes. For example, there is evidence that increases in brain manganese are larger when exposures occur via nasal instillation or inhalation than via other routes (oral or i.p.), even at the same blood manganese levels (Roels et al 1997), or even in the absence of a measured increase in blood manganese levels (Vitarella et al 2000). Notably though, the observations of a consistent manganese effect on striatal GABA levels over the whole range of cumulative dose, and an emerging effect of manganese on striatal dopamine levels at medium to high doses are equally supported by the evidence presented here, even if inhalation studies, which are a minority, are excluded from the analysis.

While the cumulative dose metric reflects both nominal dose and exposure duration, only nominal doses that exceed homeostatic control processes for a biologically important duration of time would be expected to produce toxicity, while lower nominal doses would not. This can be illustrated with an example of human dietary manganese intake. A safe internal cumulative dose of ~40mg Mn/Kg can be calculated assuming lifetime exposure to the recommended daily dietary manganese intake (ATSDR 2000). This cumulative dose does not produce signs of toxicity because the nominal dose is readily managed through homeostatic regulation. However, this estimated safe internal cumulative dose is higher than the cumulative doses shown to produce measurable toxicity in many of the rodent and non-human primate studies evaluated here (Figure 1, 2). Clearly, the homeostatic capacity was exceeded in these animal studies because when reported, brain manganese concentrations were always elevated, even in the studies with the lowest cumulative doses (Bull 1978; Chandra and Shukla 1981; Normandin et al 2002; Salehi et al 2003; Subhash and Padmashree 1991; Tapin et al 2005). Similarly, in monkey studies where adverse effects were detected below 40 mg Mn/Kg, manganese administration was via several high manganese content injections that most likely exceeded homeostatic regulation and produced elevated brain manganese levels (Newland et al 1989). Thus, while cumulative dose may be predictive of toxicity in many cases of elevated exposures, the dosing regimen (nominal dose, frequency and duration of dosing) are also important to consider when evaluating the manganese dose – response relationship. For example, in non-inhalation studies, monkeys were given

manganese in a few single acute injections separated by relatively long periods with no exposure and remarkably, only these non-inhalation studies detected motor effects due to manganese, whereas continuous inhalation studies did not (Bird et al 1984; Ulrich et al 1979a, 1979b, 1979c; Van Bogaert and Dallemagne 1946)

Summary

Rodent and non-human primate animal model studies of manganese neurotoxicity utilized cumulative manganese doses that collectively range over more than three orders of magnitude. A relatively consistent effect of manganese on motor function and striatal GABA levels is apparent across a wide range of cumulative doses. A progression of effects was noted from changes in the GABAergic system to changes in the dopaminergic system as a function of increasing cumulative dose, leading to dopamine depletion. Notably, the range of cumulative doses employed across the studies exceeds by several orders of magnitude the cumulative doses shown to produce motor disturbances in occupationally exposed humans. In light of this, there is a need for a new generation of animal model studies that employ manganese exposure regimens more relevant to the low to moderate levels of chronic lifetime environmental exposures expected from MMT emission products or other environmental sources of airborne manganese. In order to arrive at protective standards for future exposure, inhalation studies that use the same manganese compounds to

be found in the environment, such as manganese sulfate, manganese phosphate and the manganese oxide hausmannite may be most appropriate.

FIGURE CAPTIONS

Figure 1: Motor, histological, and neurochemical outcomes from rodent studies of manganese toxicity. A dash indicates that the endpoint was measured but a manganese effect was not detected. A blue inverted triangle indicates a decrease and a red triangle an increase in the particular outcome due to manganese exposure, relative to control. Manganese species given, route of administration, and animal species are listed to the right (i.p., intraperitoneal). The shaded bin indicates the lowest cumulative manganese doses where neurological effects were detected in humans in occupational settings (Roels et al 1992; Lucchini et al 1995). * Brain manganese levels of control and manganese treated animals were both higher than those of any manganese treatment from any other rodent study reviewed here. Hureaulite chemical composition is $Mn(II)_5(PO_4)_2(PO_3(OH))_2.4H_20$.

Figure 2: Behavioral/motor, histological, and neurochemical measures of non-human primate studies of manganese toxicity. A dash indicates the endpoint was measured but a manganese effect was not detected, a red triangle indicates an increase, an inverted blue triangle a decrease or a negative impact, and a diamond indicates the presence of a non-directional effect in the particular outcome due to manganese exposure. The manganese species given, route of administration (s.c., subcutaneous; i.m., intramuscular; i.v., intravenous; i.p., intraperitoneal), and animal species are listed to the right. The shaded bin indicates the lowest cumulative doses where neurological effects were detected

in occupationally exposed humans (Roels et al 1992; Lucchini et al 1995). Short horizontal arrows indicate the lowest cumulative dose given in the study as a result of the first manganese injection or initiation of inhalation. Outcomes to the right of the vertical line were produced by MnO_2 exposure and almost all outcomes to the left by $MnCl_2$ exposure. Only Bird et al. (1984), Eriksson et al 1992a, Neff et al. (1969), Ulrich et al (1979a, 1979 b, 1979c) report average outcomes for more than one animal. Reported outcomes from other studies are from single animals.

Figure 1

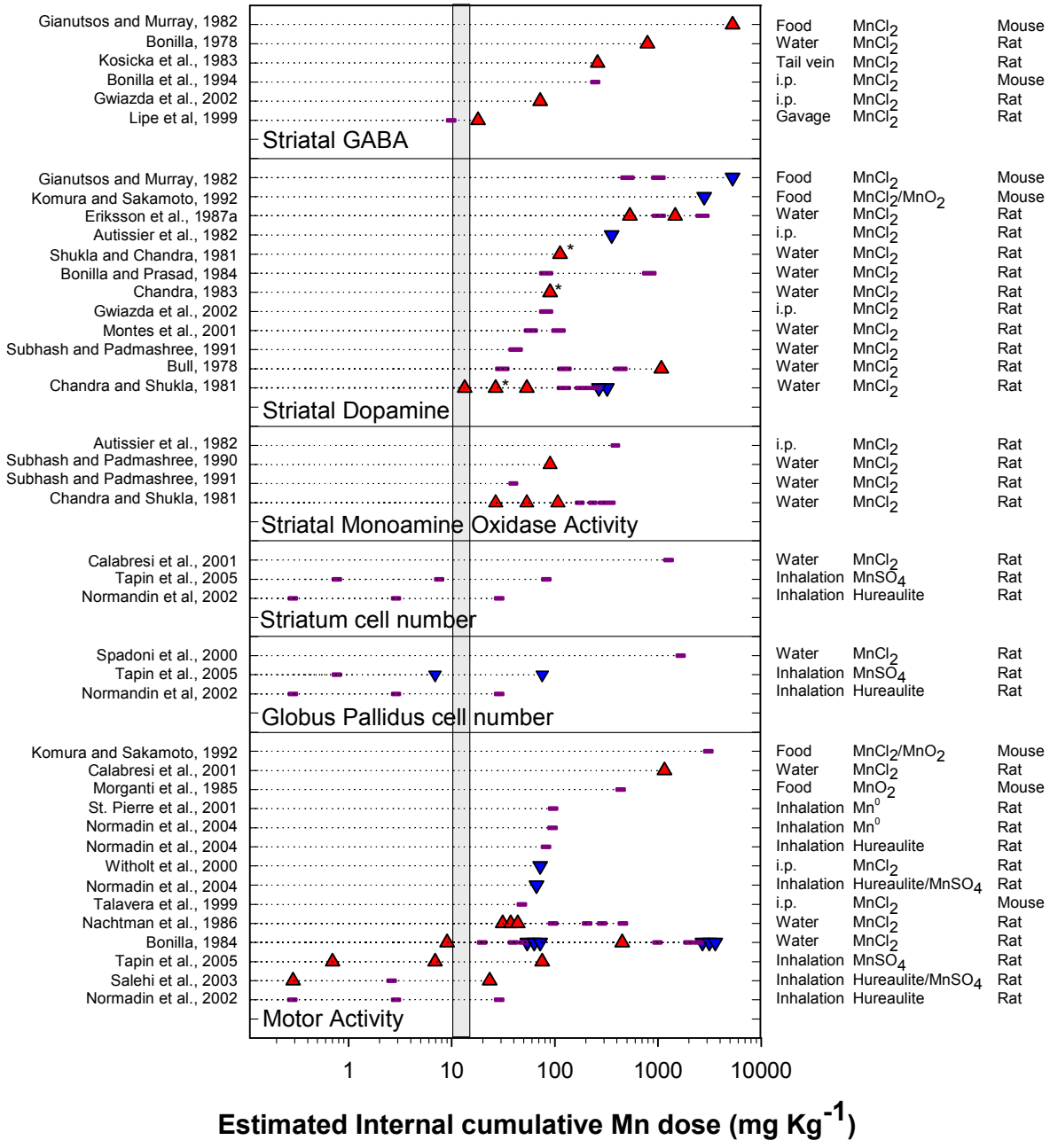
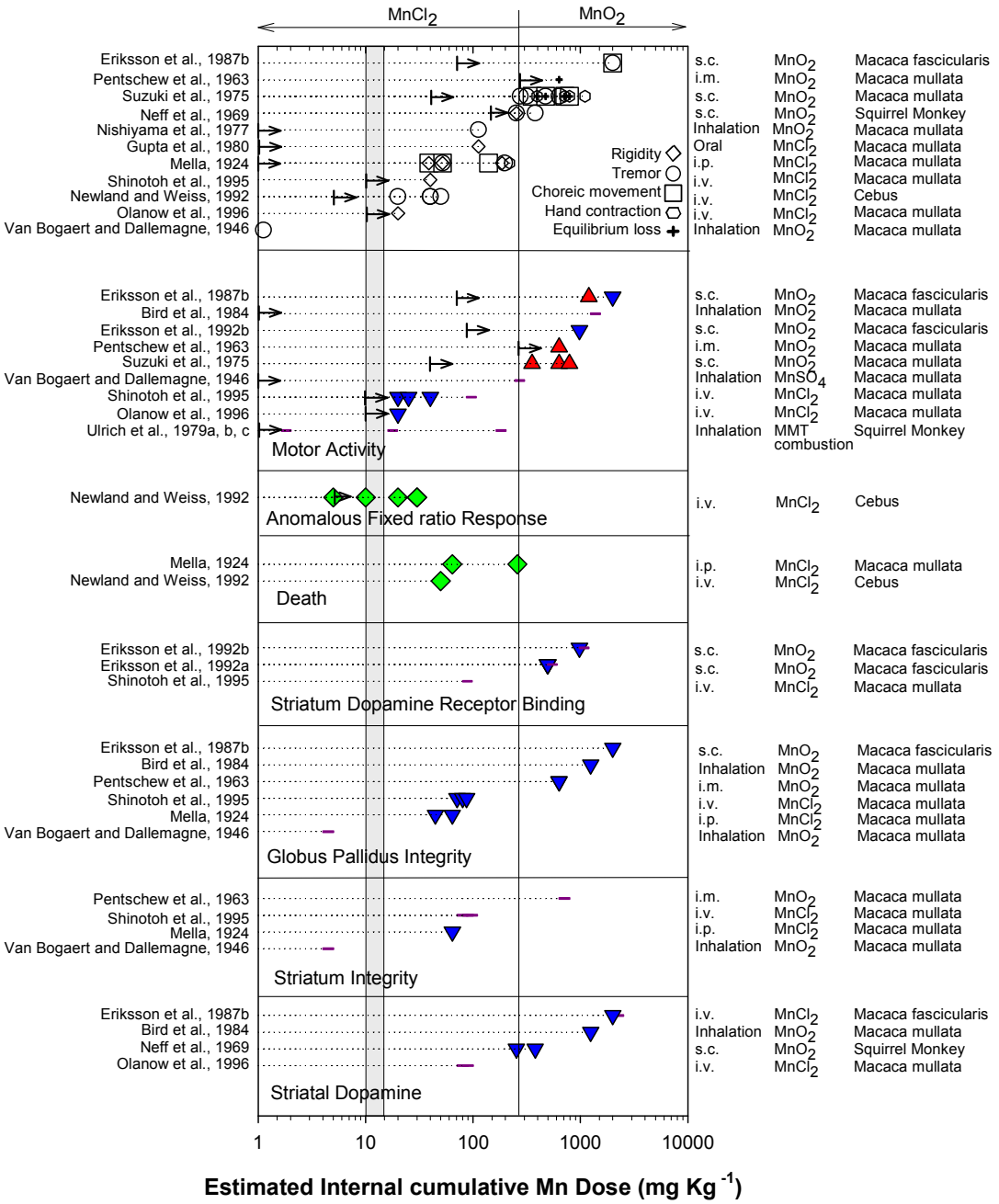

Gianutsos and Murray, 1982
Bonilla, 1978
Kosicka et al., 1983
Bonilla et al., 1994
Gwiazda et al., 2002
Lipe et al, 1999
Striatal GABA
Food MnCl2 Mouse
Water MnCl2 Rat
Tail vein MnCl2 Rat
i.p. MnCl2 Mouse
i.p. MnCl2 Rat
Gavage MnCl2 Rat
Gianutsos and Murray, 1982
Komura and Sakamoto, 1992
Eriksson et al., 1987a
Autissier et al., 1982
Shukla and Chandra, 1981
Bonilla and Prasad, 1984
Chandra, 1983
Gwiazda et al., 2002
Montes et al., 2001
Subhash and Padmashree, 1991
Bull, 1978
Chandra and Shukla, 1981
Striatal Dopamine
Food MnCl2 Mouse
Food MnCl2/MnO2 Mouse
Water MnCl2 Rat
i.p. MnCl2 Rat
Water MnCl2 Rat
Water MnCl2 Rat
Water MnCl2 Rat
i.p. MnCl2 Rat
Water MnCl2 Rat
Water MnCl2 Rat
Water MnCl2 Rat
Water MnCl2 Rat
Autissier et al., 1982
Subhash and Padmashree, 1990
Subhash and Padmashree, 1991
Chandra and Shukla, 1981
Striatal Monoamine Oxidase Activity
i.p. MnCl2 Rat
Water MnCl2 Rat
Water MnCl2 Rat
Water MnCl2 Rat
Calabresi et al., 2001
Tapin et al., 2005
Normandin et al, 2002
Striatum cell number
Water MnCl2 Rat
Inhalation MnSO4 Rat
Inhalation Hureaulite Rat
Spadoni et al., 2000
Tapin et al., 2005
Normandin et al, 2002
Globus Pallidus cell number
Water MnCl2 Rat
Inhalation MnSO4 Rat
Inhalation Hureaulite Rat
Komura and Sakamoto, 1992
Calabresi et al., 2001
Morganti et al., 1985
St. Pierre et al., 2001
Normadin et al., 2004
Normadin et al., 2004
Witholt et al., 2000
Normadin et al., 2004
Talavera et al., 1999
Nachtman et al., 1986
Bonilla, 1984
Tapin et al., 2005
Salehi et al., 2003
Normadin et al., 2002
Motor Activity
Food MnCl2/MnO2 Mouse
Water MnCl2 Rat
Food MnO2 Mouse
Inhalation Mn0 Rat
Inhalation Mn0 Rat
Inhalation Hureaulite Rat
i.p. MnCl2 Rat
Inhalation Hureaulite/MnSO4 Rat
i.p. MnCl2 Mouse
Water MnCl2 Rat
Water MnCl2 Rat
Inhalation MnSO4 Rat
Inhalation Hureaulite/MnSO4 Rat
Inhalation Hureaulite Rat
1
10
100
1000
10000
Estimated Internal cumulative Mn dose (mg Kg-1)

Figure 2


MnCl2
MnO2
Eriksson et al., 1987b
Pentschew et al., 1963
Suzuki et al., 1975
Neff et al., 1969
Nishiyama et al., 1977
Gupta et al., 1980
Mella, 1924
Shinotoh et al., 1995
Newland and Weiss, 1992
Olanow et al., 1996
Van Bogaert and Dallemagne, 1946
Rigidity
Tremor
Choreic movement
Hand contraction
Equilibrium loss
s.c. MnO2 Macaca fascicularis
i.m. MnO2 Macaca mullata
s.c. MnO2 Macaca mullata
s.c. MnO2 Squirrel Monkey
Inhalation MnO2 Macaca mullata
Oral MnCl2 Macaca mullata
i.p. MnCl2 Macaca mullata
i.v. MnCl2 Macaca mullata
i.v. MnCl2 Cebus
i.v. MnCl2 Macaca mullata
Inhalation MnO2 Macaca mullata
Eriksson et al., 1987b
Bird et al., 1984
Eriksson et al., 1992b
Pentschew et al., 1963
Suzuki et al., 1975
Van Bogaert and Dallemagne, 1946
Shinotoh et al., 1995
Olanow et al., 1996
Ulrich et al., 1979a, b, c
Motor Activity
s.c. MnO2 Macaca fascicularis
Inhalation MnO2 Macaca mullata
s.c. MnO2 Macaca fascicularis
i.m. MnO2 Macaca mullata
s.c. MnO2 Macaca mullata
Inhalation MnSO4 Macaca mullata
i.v. MnCl2 Macaca mullata
i.v. MnCl2 Macaca mullata
Inhalation MMT combustion Squirrel Monkey
Newland and Weiss, 1992
Anomalous Fixed ratio Response
i.v. MnCl2 Cebus
Mella, 1924
Newland and Weiss, 1992
Death
i.p. MnCl2 Macaca mullata
i.v. MnCl2 Cebus
Eriksson et al., 1992b
Eriksson et al., 1992a
Shinotoh et al., 1995
Striatum Dopamine Receptor Binding
s.c. MnO2 Macaca fascicularis
s.c. MnO2 Macaca fascicularis
i.v. MnCl2 Macaca mullata
Eriksson et al., 1987b
Bird et al., 1984
Pentschew et al., 1963
Shinotoh et al., 1995
Mella, 1924
Van Bogaert and Dallemagne, 1946
Globus Pallidus Integrity
s.c. MnO2 Macaca fascicularis
Inhalation MnO2 Macaca mullata
i.m. MnO2 Macaca mullata
i.v. MnCl2 Macaca mullata
i.p. MnCl2 Macaca mullata
Inhalation MnO2 Macaca mullata
Pentschew et al., 1963
Shinotoh et al., 1995
Mella, 1924
Van Bogaert and Dallemagne, 1946
Striatum Integrity
i.m. MnO2 Macaca mullata
i.v. MnCl2 Macaca mullata
i.p. MnCl2 Macaca mullata
Inhalation MnO2 Macaca mullata
Eriksson et al., 1987b
Bird et al., 1984
Neff et al., 1969
Olanow et al., 1996
Striatal Dopamine
i.v. MnCl2 Macaca fascicularis
Inhalation MnO2 Macaca mullata
s.c. MnO2 Squirrel Monkey
i.v. MnCl2 Macaca mullata
1
10
100
1000
10000
Estimated Internal cumulative Mn Dose (mg Kg -1)

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