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Rotenone and paraquat perturb dopamine metabolism: a computational analysis of pesticide toxicity

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Abstract

Pesticides, such as rotenone and paraquat, are suspected in the pathogenesis of Parkinson’s disease (PD), whose hallmark is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Thus, compounds expected to play a role in the pathogenesis of PD will likely impact the function of dopaminergic neurons. To explore the relationship between pesticide exposure and dopaminergic toxicity, we developed a custom-tailored mathematical model of dopamine metabolism and utilized it to infer potential mechanisms underlying the toxicity of rotenone and paraquat, asking how these pesticides perturb specific processes. We performed two types of analyses, which are conceptually different and complement each other. The first analysis, a purely algebraic reverse engineering approach, analytically and deterministically computes the altered profile of enzyme activities that characterize the effects of a pesticide. The second method consists of large-scale Monte Carlo simulations that statistically reveal possible mechanisms of pesticides. The results from the reverse engineering approach show that rotenone and paraquat exposures lead to distinctly different flux perturbations. Rotenone seems to affect all fluxes associated with dopamine compartmentalization, whereas paraquat exposure perturbs fluxes associated with dopamine and its breakdown metabolites. The statistical results of the Monte-Carlo analysis suggest several specific mechanisms. The findings are interesting, because no a priori assumptions are made regarding specific pesticide actions, and all parameters characterizing the processes in the dopamine model are treated in an unbiased manner. Our results show how approaches from computational systems biology can help identify mechanisms underlying the toxicity of pesticide exposure.

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Keywords
dopamine; mathematical model; mode of action; paraquat; Parkinson’s disease; rotenone

1. Introduction

Pesticides, such as rotenone and paraquat, have been suggested as contributors to the pathogenesis of Parkinson’s disease (PD), the 2nd most common neurodegenerative disorder (Brown et al. 2006; Costello et al. 2009; Giasson and Lee 2000; Gorell et al. 1998; Le Couteur et al. 1999; Priyadarshi et al. 2000; Tanner and Goldman 1996; Wang et al. 2011). In animal models, paraquat exposure can cause a loss of dopaminergic neurons and lead to an aggregation of α–synuclein (Brooks et al. 1999; McCormack et al. 2002), and rotenone exposure can reproduce many of the typical features of PD (Betarbet et al. 2000; Sherer et al. 2001; Sherer et al. 2003). Although the association between pesticide exposure and PD has been established, the actual, specific impacts of pesticides on dopaminergic neuron function are not clear. Paraquat has a chemical structure similar to the neurotoxin 1-methyl-4-phenylpyridinium ion (MPP+), a reaction product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is primarily known as an impurity of some illicitly manufactured recreational drugs (Kopin 1987). Based on this chemical similarity, paraquat toxicity has been attributed to its inhibitory effect on mitochondrial complex I (Cocheme and Murphy 2008). However, it has been argued that paraquat toxicity is not mediated through the dopamine transporter (DAT), which is required in MPP+ induced loss of dopaminergic neurons (Richardson et al. 2005). Moreover, paraquat is not a substrate for DAT in its native divalent cation state (Rappold et al. 2011), and it is only a very weak inhibitor of mitochondrial complex I with an IC50 of 8.1 mM (Richardson et al. 2005).

Rotenone can also inhibit mitochondrial complex I (Betarbet et al. 2000; Sherer et al. 2001). However, Betarbet and coworkers (2000) demonstrated that rotenone exerts uniform inhibition of mitochondrial complex I throughout the brain. In consideration of the distinctive dopaminergic neuronal loss in PD, these observations suggest that mitochondrial complex I inhibition cannot fully explain the preferential toxicity of paraquat and rotenone.

A hallmark of PD is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Thus, for paraquat and rotenone to be causative of PD, they need to specifically target dopaminergic neurons, either directly or indirectly. Mitochondrial complex I inhibition by rotenone and paraquat may induce cell apoptosis, but it lacks the specificity of targeting dopaminergic neurons. Rotenone specifically targets human dopaminergic SH-SY5Y cells, but not breast cancer MCF-7 cells and hepatoma HepG2 cells, although it inhibits mitochondrial complex I in all these cells and produces reactive oxygen species (Greenamyre et al. 2003; Rowlands and Casida 1998; Watabe and Nakaki 2007). To establish specific toxicity patterns of pesticide exposure in dopaminergic neurons, recent attention has focused on dopamine metabolism. Watabe and Nakaki used human dopaminergic SH-SY5Y cells to investigate the association between dopamine metabolism and rotenone-induced apoptosis (Watabe and Nakaki 2007). They proposed that the dopamine redistribution from vesicles to the cytosol may account for rotenone toxicity.

Sakka and collaborators (2003) suggested that dopamine mediates rotenone selective toxicity in the mesencephalon. Rotenone was furthermore suggested to inhibit the enzyme tyrosine hydroxylase (TH), which is the rate limiting enzyme of dopamine synthesis (Hirata and Nagatsu 2005). The specific toxicity of MPP+ in dopaminergic neurons seems to be associated with the utilization of DAT; however, DAT does not mediate rotenone toxicity, although both toxins (MPP+ and rotenone) inhibit mitochondrial complex I (Hirata et al. 2008). Sai and coworkers (2008) proposed that rotenone alters dopamine distribution and metabolism, leading to its selective toxicity in dopaminergic neurons. Similarly, Lawal and
colleagues (2010) suggested that rotenone, but not paraquat, targets dopamine storage, with toxic consequences at least in Drosophila. The toxic mechanisms of paraquat may be related to oxidative damage through promoting superoxide and hydrogen peroxide, which are normal by-products of dopamine metabolism (Richardson et al. 2005).

To study pesticide action in dopaminergic neurons, we utilize here a computational approach to infer sites within the dopamine pathway system that are potentially targeted by rotenone and paraquat. In contrast to traditional, targeted experimentation, computational systems biology does not necessarily rely on the a priori formulation of a specific hypothesis, and often investigates biological questions from a systemic point of view with the help of dynamic models. Over the past years we have developed such models to investigate dopamine homeostasis and dynamics in dopaminergic neurons, as well as dopamine-based signal transduction across synapses (Qi et al. 2008, 2009, 2010a, b, 2011a, 2013). These models can serve as computational platforms for simulations of dopamine synthesis, transport, release, degradation, and reuptake. They can also be utilized to identify “choke points” that are particularly vulnerable to perturbations. In addition, these models have been applied to study dopamine related diseases.

In the present study, we describe how a mathematical model of dopamine metabolism may be used to investigate the specific effects of paraquat and rotenone. While it is clear that pesticides could have multiple aspects of PD-related toxicity, we focus here specifically on perturbations of dopamine metabolism in dopaminergic neurons. Our first approach does not involve an a priori hypothesis and is directly based on a top-down analysis of experimental observations characterizing the effects of pesticides on dopamine metabolism. Like with other mathematical models, this approach is employed to obtain unique answers. As a second, complementary approach, we use a Monte Carlo simulation method that reveals potential pesticide action sites in a specific and statistically robust manner. Our findings are predictive and may serve as a basis for guiding and targeting future biological studies of the impacts of pesticides on dopaminergic neurons.

2. Methods

2.1. A mathematical model of dopamine metabolism

Over the past years, we have been developing and refining a series of mathematical models of dopamine metabolism, dopamine-associated signal transduction, and the effects of disease or drug use on normal functioning (Qi et al. 2008, 2009, 2010a, b, 2011a,b, 2012; Voit et al. 2008, 2012; Wu et al. 2011). One of these models serves as the computational platform for the present study; details regarding the dopamine pathway structure and the description of equations have been presented elsewhere (Qi et al. 2008, 2012).

In a nutshell, dopamine is synthesized from the precursor L-DOPA, which is produced from the essential amino acid tyrosine that is made available to the brain through the bloodstream. Synthesized dopamine is packed into storage vesicles through the vesicular monoamine transporter VMAT2. Spontaneously, or in response to a stimulus, vesicular dopamine is released into the synaptic cleft where it executes its signaling function. Released dopamine can be carried back to the presynaptic terminal for recycling through the specific transporter DAT. Dopamine can also be enzymatically converted into other metabolites such as 3,4-dihydroxyphenylacetae (DOPAC) and homovanillic acid (HVA). In addition to these fundamental processes, the dopamine model accounts for many secondary processes, as well as regulatory processes, such as inhibition signals that affect certain enzymatic steps.
The model is set up with ordinary differential equations (ODEs) and uses mass action and Michaelis-Menten representations for all biochemical reactions. Most of the numerical values for concentrations, turnover rates, reaction orders, and Michaelis constants were obtained from experimental measurements reported in literature. The few remaining parameters were estimated by fitting the model to experimental data. The parameterized model was tested with standard methods of algebraic and computational diagnostics, as well as a variety of simulation studies, and the reliability and correctness of the model were validated against biological and clinical observations (Qi et al. 2008, 2012).

We performed two types of analyses, which are conceptually different and complement each other. The first analysis, a purely algebraic reverse engineering approach, results in a singular prediction for the effects of a pesticide. While elegant, however, it does not offer a measure of reliability and statistical significance. It is also limited to a relatively small number of candidate processes. The second method consists of large-scale Monte Carlo simulations that lead to distributions of possible actions of pesticides, which are assessed statistically. By and large, the two methods yielded consistent results, even though this was not a priori guaranteed.

2.2. Pertinent literature information for model analysis

Both the reverse engineering method and the Monte Carlo simulations require information regarding the normal and the pesticide-affected steady states of the metabolites in the dopamine system. The two approaches then infer what alterations of processes have an effect on the system compatible with the perturbed steady-state profiles of the metabolic pathway under pesticide exposure. The normal state of the system was discussed in our earlier studies (Qi et al. 2008, 2012). For the rotenone and paraquat affected states, we used information from the literature (Kang et al. 2009; Ren et al. 2009; Watabe and Nakaki 2007, 2008). Table I describes experimental data characterizing the effects of rotenone and paraquat on the dopamine system.

2.3. Reverse engineering

This approach is based on a method that we previously developed in different contexts to reverse-engineer biochemical systems (Voit 2009, Lee et al. 2011); see also (Chiang et al. 2011). An ODEs system in the so-called S-system format can be assessed at its steady state with methods of linear algebra instead of a numerical search algorithm (Savageau 1969; Voit 2000, 2013). To utilize this advantage, we converted our model, which was originally represented with Michaelis-Menten and mass action kinetics, into an S-system representation at the normal steady state. Subsequently, we analytically and deterministically computed the altered profile of enzyme activities, which characterizes the effects of a pesticide on the dopamine system. In other words, these changes in the activities of particular enzymes or transporters and their associated rate constants are computed such that the corresponding metabolites have pesticide-affected values that are similar to experimental observations.

Due to the structure of S-system models, any solution from this method is actually comprised of a set of ratios between the rate constant for the influx and that for the efflux associated with each metabolite. Thus, the approach characterizes the overall perturbation of fluxes through metabolites caused by pesticide exposure. The main advantages of this solution are its simplicity, uniqueness, and mathematical elegance. At the same time, the method has three drawbacks. First, the method is truly effective only if the number of affected fluxes can be assumed to be the same as the number of observed changes in metabolites. Second, it provides only a snap-shot without bounds on reliability. And finally,
if no observations on specific metabolites are available, one must assume that these are unchanged, which is not always true, as we will see later in one of the results.

2.4. Monte Carlo simulations

The second strategy of our analysis is mathematically less direct, but much more flexible. It proceeds as follows. Using the mathematical model of dopamine metabolism as a platform, we execute very many Monte Carlo simulations of the model with random combinations of parameter values that are uniformly sampled from reasonable ranges. Thus, very little bias and no restrictive assumptions are hidden in these simulations. At the end of each simulation, we check whether the simulated result is sufficiently similar to the experimental findings. We declare a simulation as a good potential solution if its simulated effect on dopamine metabolism is very close to that of a pesticide. If so, the result is retained, but if not, the simulation is discarded. To determine the quality of a solution, we use the Euclidean distance between the simulated and the observed metabolic profiles under pesticide exposure. Specifically, we use a normalized distance of 0.3 as the upper bound for admissible solutions. This value actually corresponds to a rather loose criterion that allows considerable noise in the selected solutions and thereby makes the inferred mechanisms of pesticides that are deemed significant more statistically reliable than a more stringent criterion would. Following the initial results, we alter the criterion to compare results obtained with different admissible distance bounds to assess the consistency and robustness of the results.

For each admissible solution, each pertinent feature (parameter value) is added to a corresponding (“posterior”) frequency distribution that becomes more reliable with each successful simulation. Thus, the ultimately retained results consist of one posterior distribution for each parameter in the dopamine system. Expressed differently, each parameter value in each distribution is, in combination with other valid parameter values, consistent with experimental findings. Ultimately, the simulation settings surviving this filtering can be interpreted as valid model instantiations for cells exposed to a pesticide.

If a site is not substantially targeted by a pesticide, its posterior distribution among all simulations mimicking pesticide toxicity should be uniform or maybe Gaussian about some nominal value. By contrast, a posterior distribution that is strongly skewed reveals settings of a parameter that are likely consistent (high frequency of values) or inconsistent (low frequency) with the available experimental data. Thus, we can statistically predict mechanisms underlying pesticide toxicity in dopaminergic neurons from a systemic point of view.

2.5. Statistical analysis

In order to establish a measure of reliability in our conclusions, we use the bootstrap method (Manly 2006). We randomly sample 250,000 solutions from among the total of 500,000 simulations, and, by repeatedly sampling, form 100 sub-datasets. These sub-datasets are used to determine with statistical significance if the activity of an enzymatic or transport process is activated or inhibited by the pesticide. To quantify these results, we generate a distribution of the ratios between the area for activation and that for inhibition. For easier interpretation, the inverse ratios are used where the mean value of ratios is less than 1. In this analysis, we assess statistical significance with a p-value less than 0.001 in a paired t-test; this significance threshold is denoted in figures with three asterisks.
3. Results

3.1. Mechanisms of pesticides inferred through reverse engineering

The reverse engineering approach was directly applied to the model (in S-system representation) in order to pinpoint potential effects of rotenone and paraquat on the fluxes in the dopamine system. The results show that rotenone and paraquat exposures lead to distinctly different flux perturbations (Fig. 1). According to this analysis, rotenone (panel A) affects all fluxes associated with the compartmentalization of dopamine among cytosol, vesicles, and the synaptic cleft. In particular, the flux of dopamine flowing into the synaptic cleft (marked as eDA), which is the carrier of input signals to the postsynapse, is strongly increased, whereas the releasable dopamine stored in vesicles (IDA) exhibits a reduced overall flux. The pathway map in Fig. 2A shows metabolites associated with through-fluxes affected by rotenone.

In contrast to the compartmentalization of dopamine targeted by rotenone, paraquat exposure (Fig. 1, panel B) perturbs fluxes associated with dopamine and its breakdown metabolites, such as cytosolic and extracellular 3,4-dihydroxyphenylacetate (DOPAC) and homovanillate (HVA); these affected fluxes are also shown in Fig. 2B). Specifically, paraquat elevates fluxes into DOPAC and suppresses the flux toward HVA. Interestingly, paraquat exposure does not seem to perturb the flux of dopamine in and out of the synaptic cleft.

3.2. Mechanisms of pesticides inferred from Monte Carlo Simulations

Although no a priori assumptions regarding specific mechanisms of pesticide toxicity were made, and all parameters (Michaelis constants, maximum rates, rate constants) characterizing the processes in the dopamine model were treated in an unbiased manner, the results of the Monte-Carlo analysis suggest several specific mechanisms of pesticide toxicity in dopaminergic neurons as highly likely. As discussed in the Methods section, uniform distributions are not informative, whereas strongly skewed distributions point to likely mechanisms of pesticide toxicity.

Figure 3 shows the simulated distributions of parameter values for all processes in the dopamine system under rotenone exposure; the corresponding results for paraquat are shown in Figure S1 of the Supplements. Out of 500,000 Monte Carlo simulations, only those results were retained that match experimental findings within an admissible bound, as described in the Methods section. As one can see, several parameters exhibit strongly skewed posterior distributions, indicating that these are candidate targets of rotenone. Of particular note are parameters associated with the capacity of tyrosine hydroxylase, the capacity of VMAT2, and the rate of dopamine release.

Figure 4 shows details of the distributions of the three parameters that are most strongly affected by rotenone and paraquat, based on 500,000 Monte Carlo simulations. The left column refers to a control (the maximum rate of the enzyme dopachrome isomerase $V_{\text{max-dct}}$) which, according to observations, is unaffected by the pesticides and used for comparison. As one might expect, the posterior distribution of this control is essentially uniform. The remaining three columns represent the maximum transport rate of VMAT2 ($V_{\text{max-vmat2}}$), $V_{\text{max}}$ of tyrosine hydroxylase ($V_{\text{max-th}}$), and the rate constant for dopamine release ($K_{\text{release}}$).

The results in Figure 4A indicate that the maximum rate for the transporter VMAT2 is reduced under rotenone exposure. In fact, almost no combination of parameter values with an increased $V_{\text{max-vmat2}}$ value survived the filtering against rotenone data. Since half a million cases were tested and a rather loose criterion of selection was applied, this result is
quite consistent and strong. Furthermore, the rate constant for dopamine release into the cleft is elevated, compared with the control (left column of Fig. 4A). Again, this result is rather clear, as only very few simulations with a reduced release rate survived the filtering; these may be considered as noise. Both results confirm, and refine, the result from the reverse engineering analysis. In addition, the maximum turnover rate $V_{\text{max-th}}$ for the rate-limiting enzyme TH seems to be mildly targeted by rotenone, since its distribution is different from the control (Fig. 3A). However, one cannot unambiguously identify the exact action of rotenone at this site because this mechanism is not as clearly associated with a skewed posterior distribution as the other two.

In the case of paraquat, the same parameters are affected, but in a different manner (Fig. 4B). In stark contrast to rotenone, the maximum turnover rate $V_{\text{max-th}}$ of the rate-limiting enzyme TH is inhibited, which one might expect to result in an overall lower synthesis of dopamine. At the same time, the rate constant for dopamine release is noticeably elevated. Finally, the maximum transport rate for the transporter VMAT2 could be targeted by paraquat, but the effect is not obvious and will require further analysis.

The Monte Carlo strategy does not exclude the possibility of interdependencies between parameters. In other words, a parameter value may only be admissible if the value of another parameter falls within a certain range. To reveal such potential parameter interdependencies, we constructed 3D scatter plots of the values of the three significant parameters ($V_{\text{max-vmat2}}$, $V_{\text{max-th}}$, and $K_{\text{release}}$) that were suggested by the Monte Carlo simulations as strongly affected by either rotenone or paraquat (Fig. 5). Both scatter plots constitute compact domains that do not show particular orientations or shapes that would suggest parameter interdependencies. This result suggests that there might be a certain degree of compensation among parameters but that the inferred parameters are individually significant targets of pesticides, even if other parameter values vary within reasonable ranges.

In addition to qualitatively screening for action sites of pesticides, the Monte Carlo method provides a measure of intensity of the actions, along with statistical information, such as standard deviations (Table II). Methodological comparison shows that results from the Monte Carlo simulations and the reverse engineering approach are mostly consistent. However, the Monte Carlo method points to an additional, strong effect of paraquat on the enzyme TH, which was missed by the reverse engineering approach, because no observations regarding the metabolite DOPA were available. Thus, the Monte Carlo method, while much more computationally intensive, is more flexible and inclusive by addressing every parameter in the system, even if data are scarce. Nevertheless, one must caution that it is possible that the Monte Carlo method misses underlying mechanisms and that data scarcity limits the predictive power of the model. We will return to this aspect in the Discussion.

### 3.3. Analyses of uncertainty in Monte Carlo results

We analyzed the reliability of each identified mechanism of pesticide toxicity as revealed by the Monte Carlo simulations. Two factors might affect these results and the reliability of the discovered mechanisms. One is the simulation scenario itself. For Figure 4, the simulation number is 500,000, which is quite large. Thus, one might ask whether the same mechanisms would be identified or whether some new mechanisms could appear, if the number of simulations were greatly changed. To address this issue, we tested different simulation scenarios with 50,000, 100,000, and 200,000 simulations. As shown in Figure 6, all four simulation scenarios in the case of rotenone exposure lead to the same inference of the mechanisms, that is, the same sites and modes of action, as before. As expected, simulations with higher numbers lead to smoother posterior distributions. The same result holds for paraquat (Fig. S2).
The second potential cause of uncertainty is related to the criterion that is used to determine if a simulation mimics the actions of a pesticide and is admissible. It is quite evident that different thresholds for admissible differences, in terms of the Euclidean distance between experimental data and simulation results, allow different levels of uncertainty in the selected set of parameter values (see *Methods* section). Expressed differently, the thresholds correspond to differently sized sub-spaces in the high-dimensional space of parameter values. If the inferred sites and modes of action are truly the targets of pesticides, they should persist even under the most stringent thresholds, even if the system is affected by noise. To investigate the influences of admissibility thresholds on our results, we explored a series of upper bounds for the maximally permitted distance between experimental data and simulation results and considered solutions located within these ranges as different sets of admissible candidate solutions. This type of analysis assesses the consistency and robustness of the inferred mechanisms of pesticide toxicity. For rotenone, this analysis again led to the same mechanisms, with the same sites and modes of action (Table III, based on 500,000 simulations). The same consistency held for paraquat (Table S1).

Figure 4 suggests that TH may be targeted by rotenone and that the transporter VMAT2 may also be an action site of paraquat. These two mechanisms are not as unambiguously involved as others, because the skewedness of their distributions is not quite as obvious and strong. We therefore further analyzed these possible mechanisms of pesticide toxicity by quantitative comparisons of the solution space for increasing (activation) and decreasing (inhibition) effects of a pesticide.

To quantify these differences, we not only count the number of solutions indicating activation and that indicating inhibition, but also account for the intensity of activation or inhibition. This analysis is accomplished by computing the ratios of the areas for activation to the corresponding areas for inhibition within the posterior distributions in bootstrap samples, as described in the *Methods* section. The results of this analysis reveal that different mechanisms are associated with rotenone and paraquat not only qualitatively but also quantitatively (Fig. 7). For rotenone, the primary mechanism appears to be the inhibition of the transporter VMAT2, followed by the activation of dopamine release. The activation of TH is supported with statistical significance ($p<5.0! 10^{-139}$, paired *t*-test), although the mechanism is apparently less important than the other two mechanisms, since action on this site is not quite as skewed toward to activation or inhibition. The situation is different for paraquat. Here, the inhibition of TH emerges as the most important mechanism, while the activation of dopamine release is secondary. Although the inhibition of the transporter VMAT2 is statistically significant ($p<9.0! 10^{-139}$, paired *t*-test), it is much less likely targeted by paraquat in comparison to the other two sites.

### 4. Conclusions and Discussion

Pesticides like paraquat and rotenone have been widely used around the world for many years (Shimizu et al. 2003). Exposure to these pesticides has been associated with PD risk in many studies (Le Couteur et al. 1999; Priyadarshi et al. 2000; Semchuk et al. 1992; Tanner and Goldman 1996; Tanner and Langston 1990). However, the underlying mechanisms of chronic pesticide exposure for increased PD risk are still unclear. Some hypotheses have been made, which include inhibition of mitochondrial complex I, oxidative damage, and dopamine redistribution. Since the progressive loss of dopaminergic neurons in SNpc is a hallmark of PD, the toxicological actions of paraquat and rotenone should ultimately converge on dopaminergic neurons. For their specific toxicity, dopamine metabolism instead of mitochondrial complex I inhibition could be a critical target as suggested by some studies (Lawal et al. 2010; Sai et al. 2008; Sakka et al. 2003; Watabe and Nakaki 2007).
Dopamine metabolism constitutes a complex biochemical system, which includes synthesis, storage, release, reuptake, and degradation of the neurotransmitter (Qi et al. 2008). In addition, dopamine metabolism involves many enzymes, transporters, and regulators, so that intuitive associations between pesticides and target sites are very difficult to determine. Similarly, it seems very challenging to investigate so many components through direct experiments, which would be very time-consuming and expensive. These challenges imply that a computational screening strategy could be very helpful. The computational analysis is cheap, and millions of simulations may be performed within a relatively short period of time. More importantly, the computational screening does not necessarily require the a priori formulation of a specific hypothesis or many data as input. As demonstrated here, the approach is viable with a partially observed response of the biochemical profile of dopamine metabolism to pesticide challenges and can nevertheless generate novel predictions. The systems biological approach does not compete with experimental approaches but instead can serve as a complementary method that guides and focuses attention onto specific targets.

With rather limited information, the computational approaches proposed here provided useful insights into a complex biological system. Specifically, they predicted different mechanisms for paraquat and rotenone toxicity. According to our results, rotenone primarily inhibits the transporter VMAT2 and secondarily activates dopamine release that redistributes dopamine from the vesicle pool to the synaptic cleft and cytosol. In the case of paraquat, the inhibition of TH emerges as the most significant mechanism, while the activation of dopamine release is secondary. Thus, paraquat not only disturbs the overall biosynthesis of dopamine but also affects the distribution of dopamine among compartments.

The Monte Carlo method we proposed is quite powerful, and its results and predictions have quantifiable statistical reliability. Nonetheless, the results of course do not always tell the whole truth. For instance, if data are scarce, the method may miss mechanisms that act on some of the system variables or processes without significantly influencing the observations.

An interesting feature and advantage of the Monte Carlo approach is the following. In addition to direct modes of action, the method can capture significant actions by a pesticide even if the action site is not directly observed through experiments. An example of such an indirect target is the inferred strong effect of paraquat on the enzyme TH.

The model based predictions are not only computationally sound and statistically significant but are in some cases supported by biological experiments documented in the literature. For example, dVMAT over-expression protects dopaminergic neurons against cell loss caused by rotenone in drosophila, but doesn’t counteract paraquat toxicity (Lawal et al. 2010). Ramachandiran et al. (2007) suggested that it is likely that paraquat and rotenone have different cellular targets, which is in line with our results. Lawal et al. (2010) also showed the limitations of VMAT’s neuroprotective effects in flies exposed to paraquat and suggested differences between the neurotoxic mechanisms of paraquat versus rotenone. The analysis furthermore suggests the inhibition of TH as a significant mechanism of paraquat toxicity. According to a PubMed search, this target has not been studied directly. However, Statthakis et al. (1999) found that TH is dominantly activated by loss-of-function catecholamines-up (Catsup) mutations in Drosophila, and loss-of-function Catsup mutations elevate DA levels and are dominantly neuroprotective against paraquat exposure (Chaudhuri et al. 2007). Since there is still no clarity regarding the mechanisms of paraquat, the targets suggested by our results may be worth experimental investigations.

Specifically, biological experiments could be designed to provide deeper insights into mechanisms of pesticide toxicity. For example, one could ask the following questions,
which are directly suggested by our results and predictions: Does rotenone, but not paraquat, mainly target VMAT in mammals? In *Drosophila*, is TH targeted by paraquat? Is VMAT a more important target than TH for rotenone toxicity? Is TH a more important target than VMAT for paraquat toxicity? Could TH inhibition further increase the protection of dVMAT over-expression against rotenone toxicity? Is there synergism between TH and VMAT2 under paraquat exposure? Answering such specific biological questions, arising from our computational results, could yield a deeper understanding of pesticide mechanisms, not only qualitatively but also quantitatively.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations**

- **PD** Parkinson’s disease
- **MPP+** 1-methyl-4-phenylpyridinium ion
- **DAT** dopamine transporter
- **MPTP** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
- **SNpc** *substantia nigra pars compacta*
- **DOPAC** 3,4-dihydroxyphenylacetate
- **HVA** homovanillic acid
- **ODEs** ordinary differential equations
- **DOPAC** 3,4-dihydroxyphenylacetate
- **Catsup** catecholamines-up

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Highlights

Pesticides are suspected to contribute to the pathogenesis of Parkinson’s disease
A mathematical model is used to infer mechanisms of rotenone and paraquat toxicity
Rotenone appears to affect dopamine fluxes between cellular compartments
Paraquat exposure appears to perturb fluxes associated with dopamine breakdown
Figure 1. Alterations of flux profiles under pesticide exposure, inferred through reverse engineering
The reverse engineering approach was applied to a dynamic model of dopamine metabolism in the so-called S-system representation. The results pinpoint different effects of rotenone and paraquat on the fluxes within the dopamine system. Fluxes are normalized to the corresponding fluxes under the control condition, i.e., without pesticide exposure.

A. Rotenone affects fluxes associated with the compartmentalization of dopamine among cytosol, vesicles, and the synaptic cleft. In particular, the flux of dopamine into the synaptic cleft (eDA) which is the carrier of input signals to the postsynapse, is increased, whereas the flux of releasable dopamine stored in vesicles (IDA) shows a reduced overall magnitude.

B. Paraquat exposure perturbs fluxes associated with dopamine and its breakdown metabolites, such as 3,4-dihydroxyphenylacetate (DOPAC) and homovanillate (HVA). Specifically, paraquat elevates fluxes into cytosolic and external DOPAC and suppresses the flux toward HVA. Interestingly, paraquat exposure does not seem to perturb the flux of dopamine through the synaptic cleft.
Figure 2. Schematic maps of pesticide action inferred through reverse engineering
Red arrows indicate an elevated overall flux through the corresponding metabolite due to pesticide exposure, while green arrows indicate reduced overall fluxes. A. Rotenone exposure; B. Paraquat exposure.
Figure 3. Distributions of parameters in dopaminergic neurons under rotenone exposure
Simulated distributions of parameter values for all processes in the dopamine system under rotenone exposure are shown. Out of 500,000 Monte Carlo simulations, only those results were retained that match experimental findings within a small error band. Several parameter values exhibit strongly skewed posterior distributions, indicating that they are candidate targets of rotenone.

Sub-panel titles refer to the following kinetic parameters: P1: $V_{\text{max-tyrosine}}$; P2: $K_{\text{tyrosine-tyrosine buffer}}$; P3: $V_{\text{max-th}}$; P4: $V_{\text{max-aadc}}$; P5: $V_{\text{max-vmat2}}$; P6: $K_{\text{release}}$; P7: $V_{\text{max-dat}}$; P8: $V_{\text{max-leakage-dat}}$; P9: $V_{\text{max-glialuptake}}$; P10: $V_{\text{max-comt}}$; P11: $K_{\text{3mt-hva-mao}}$; P12: $K_{\text{dopamine-dopac-maoaldh}}$; P13: $K_{\text{glialdopamine-glialdopac-maoaldh}}$; P14: $K_{\text{dopac-hva-comt}}$; P15: $K_{\text{dopac-removal}}$; P16: $K_{\text{glialdopac-hva-comt}}$; P17: $K_{\text{glialdopac-removal}}$; P18: $K_{\text{hva-removal}}$; P19: $K_{\text{dopamine-dopaminequinone}}$; P20: $K_{\text{dopaminequinone-dihydroxyindole-mif}}$; P21: $K_{\text{dihydroxyindole-melanin-tyr}}$; P22: $V_{\text{max-tyr-dopa}}$; P23: $K_{\text{dopaquinone-dopachrome}}$; P24: $K_{\text{dopachrome-dihydroxyindole-tyr}}$; P25: $V_{\text{max-dct}}$; P26: $K_{\text{melanin-removal}}$; P27: $K_{\text{extracellular dopamine-extracellular dopac-maoaldh}}$; P28: $K_{\text{extracellular dopac-hva-comt}}$; P29: $K_{\text{extracellular dopac-removal}}$.

The corresponding results for paraquat are shown in Figure S1 of the Supplements.
Figure 4. Most significant mechanisms associated with toxicity of rotenone and paraquat in dopaminergic neurons

The distributions of three kinetic parameters that are most strongly affected by rotenone or paraquat (500,000 Monte Carlo simulations) are shown. The left column (maximum rate of the enzyme dopachrome isomerase $V_{\text{max-dct}}$) is used as a control, because it is unaffected by the pesticides, according to our results. The remaining three columns (from left to right) represent the maximum transport rate of VMAT2 ($V_{\text{max-vmat2}}$), $V_{\text{max}}$ of tyrosine hydroxylase ($V_{\text{max-th}}$), and the rate constant for dopamine release ($K_{\text{release}}$).

For rotenone (row A), the results indicate that the maximum rate for the transporter VMAT2 must be reduced. Furthermore, the rate constant for dopamine release into the cleft is elevated. In addition, the maximum turnover rate $V_{\text{max-th}}$ for the rate-limiting enzyme TH seems to be mildly targeted by rotenone.

For paraquat (row B), the same parameters are affected, but in a different manner. The maximum turnover rate $V_{\text{max-th}}$ of the rate-limiting enzyme TH is inhibited. The rate constant for dopamine release is noticeably elevated. The maximum transport rate for the transporter VMAT2 could be targeted by paraquat, but the effect is not obvious.

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Figure 5. Interdependency between parameters targeted by rotenone or paraquat in dopaminergic neurons
Locations of values of three parameters ($V_{\text{max-vmat2}}$, $V_{\text{max-th}}$, and $K_{\text{release}}$), which are strongly affected by rotenone and paraquat, are plotted in a 3D space. Parameter values are relative to their normal levels. Results are based on 500,000 Monte Carlo simulations. Each symbol represents an admissible scenario of toxicity. The results exhibit no obvious patterns of interdependency between these parameters. A: rotenone exposure; B: paraquat exposure.
Figure 6. Consistency of inferred rotenone mechanisms among different simulation scenarios
Different scenarios with 50,000, 100,000, 200,000, and 500,000 simulations were tested for the case of rotenone exposure. Rows A, B, C, and D represent 50,000, 100,000, 200,000, and 500,000 simulations, respectively. All four simulation scenarios led to the inference of the same mechanisms of toxicity, that is, the same sites and modes of action. As expected, simulations with higher numbers lead to smoother posterior distributions.
Figure 7. Quantification of pesticide action with respect to activation or inhibition
The ratios of the areas for activation to the corresponding areas for inhibition within the posterior distributions in bootstrap samples are shown for rotenone and paraquat exposure. For easier interpretation, the inverse ratios are used where the mean value of ratios is less than 1. Different mechanisms are associated with rotenone and paraquat not only qualitatively but also quantitatively. The activation of TH by rotenone is supported with statistical significance ($p<5.0 \times 10^{-139}$, paired $t$-test), although the mechanism is far less important than the other two mechanisms. The inhibition of the transporter VMAT2 by paraquat is statistically significant ($p<9.0 \times 10^{-139}$, paired $t$-test), but it is not as significant as the other two sites.

From left to right, subplots are for the maximum transport rate of VMAT2 ($V_{\text{max-vmat2}}$), $V_{\text{max}}$ of tyrosine hydroxylase ($V_{\text{max-th}}$), and the rate constant for dopamine release ($K_{\text{release}}$). Rotenone and paraquat likely inhibit VMAT2, while they activate dopamine release. However, these two pesticides show opposite actions with respect to TH. Within each subplot, two open bars are controls for rotenone (Control-1) and paraquat (Control-2), respectively; the blue bar represents rotenone exposure, while the yellow bar represents paraquat exposure. Significance is shown with *** indicating $p < 0.001$ in a paired $t$-test.
Table I

Experimentally Determined Effects of Pesticide Exposure on Metabolites Associated with Dopamine Metabolism

<table>
<thead>
<tr>
<th>Concentration Change under Rotenone Exposure (Relative to Normal Levels)*</th>
<th>Concentration Change under Paraquat Exposure (Relative to Normal Levels)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicular DA: −40%</td>
<td>Tissue Dopamine: between −20% and −50%</td>
</tr>
<tr>
<td>Cytosol DA: 45%</td>
<td>DOPAC: between no change and −40%</td>
</tr>
<tr>
<td>DOPAC: no significant change</td>
<td>HVA: between −50% and −60%</td>
</tr>
<tr>
<td>HVA: no significant change</td>
<td>3-MT: between no change and −40%</td>
</tr>
<tr>
<td>Glutathione: −30%</td>
<td>Glutathione: −45%</td>
</tr>
</tbody>
</table>

* 0.4 μM rotenone for 8h in human dopaminergic SH-SY5Y Cells (Watabe and Nakaki 2007, 2008).

# Mice were treated twice weekly with paraquat (10 mg/kg, i.p.) for three consecutive weeks; or mice in the paraquat-treated group were given oral doses of paraquat (10 mg/kg) daily for four months (Kang et al. 2009; Ren et al. 2009).
Table II

Intensity of Actions of Rotenone and Paraquat Inferred from Monte Carlo Simulations#

<table>
<thead>
<tr>
<th>Site</th>
<th>Normal value</th>
<th>Rotenone</th>
<th></th>
<th></th>
<th>Paraquat</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intensity*</td>
<td>SD^</td>
<td></td>
<td>Intensity*</td>
<td>SD^</td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$-vmat2</td>
<td>0.14 μM/s</td>
<td>-29.9%</td>
<td>0.140</td>
<td>-9.7%</td>
<td>0.273</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$-th</td>
<td>0.0347 μM/s</td>
<td>11.2%</td>
<td>0.187</td>
<td>-24.5%</td>
<td>0.179</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{\text{release}}$</td>
<td>1.512 /h</td>
<td>20.3%</td>
<td>0.196</td>
<td>14.0%</td>
<td>0.227</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# 500,000 Monte Carlo simulations.

* Percentage of change relative to normal values.

^ Standard deviation.
Mechanisms for Rotenone Toxicity are Consistently Identified under Various Criteria of Admissible Differences in Terms of Euclidean Distance between Experimental Data and Simulation Results

<table>
<thead>
<tr>
<th>Distance relative to the control value</th>
<th>Sites of action</th>
<th>Mode of action</th>
<th>Significance of sites and mode of action?</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>VMAT2 TH Release*</td>
<td>Inhibit VMAT2, activate release</td>
<td>!</td>
</tr>
<tr>
<td>95%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>90%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>85%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>80%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>75%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>70%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>65%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>60%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
<tr>
<td>55%</td>
<td>VMAT2 TH Release</td>
<td>Same</td>
<td>!</td>
</tr>
</tbody>
</table>

The control value represents the Euclidean distance specified in the Methods section.

* Release represents the rate constant for dopamine release.

# The mode of action is a reduction of the maximum rate for the transporter VMAT2 and an increase in the rate constant for dopamine release. The rate-limiting enzyme TH is targeted by rotenone; but not as strongly as the other two sites. Therefore, the site TH is not included in this analysis.