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Rasagiline, a Suicide Inhibitor of Monoamine Oxidases, Binds Reversibly to α-Synuclein

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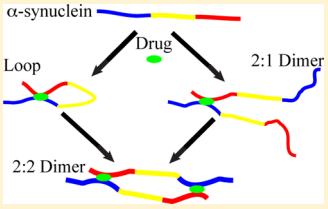
ABSTRACT: Rasagiline (*N*-propargyl-1-*R*-aminoindan) and selegiline (1-deprenyl) are MAO-B inhibitors which are used in the treatment of Parkinson's disease. The binding of rasagiline, selegiline, and their metabolites including 1-aminoindan, 2-aminoindan, and methamphetamine to α-synuclein was investigated by nanopore analysis and isothermal titration calorimetry. Blockade current histograms of α-synuclein alone give a peak at −86 pA which is due to translocation of the protein through the pore. In the presence of rasagiline and *R*-1-aminoindan, this peak shifts to about −80 pA. In the presence of selegiline and *R*-methamphetamine, the number of events at −86 pA is reduced and there is a higher proportion of bumping events at about −25 pA which are due to a more compact conformation. Rasagiline can also bind to sites in both the N- and C-terminal regions of α-synuclein. The binding constants of rasagiline and selegiline were estimated by isothermal titration calorimetry to be about 5×10^5 and $<10^4$ M^{-1}, respectively. A model is presented in which both rasagiline and *R*-1-aminoindan bind to α-synuclein, forming a loop structure which is less likely to aggregate or form fibrils. In contrast, selegiline binds and forms a more compact structure similar to that formed by methamphetamine.



KEYWORDS: *Parkinson's disease, α-synuclein, rasagiline, selegiline, neuroprotective drugs, nanopore analysis, protein folding*

Parkinson's disease (PD) was first described by an English doctor in 1817. He took extensive notes on six patients who all had symptoms of the "shaking palsy" including rigidity and slowness of movement.[1] We now know that these symptoms are caused by the death of dopamineurgic neurons in the substantia nigra which plays an important role in the motor control circuitry. Briefly, dopamine is required to release motor neurons from inhibition, and thus, when dopamine concentrations are decreased, there is a subsequent reduction in motor activity. At present, there is no cure for PD and the disease progresses in severity over the course of 5−10 years.[2] In the early stages, the motor symptoms can be alleviated by increasing dopamine concentrations either by the administration of L-DOPA which is metabolized to dopamine or by giving monoamine oxidase (MAO) inhibitors which prevent the breakdown of dopamine.[1,2] Two MAO inhibitors which have found widespread use are the propargylamines (Figure 1), rasagiline and selegiline.[3]

Both drugs have a preference for inhibition of MAO-B type enzymes rather than MAO-A, an important consideration since they are less likely to interfere with the metabolism of tyramine which is mostly degraded by MAO-A.[3,4] An increase in vascular tyramine can lead to severe hypertensive events which are exacerbated by the ingestion of tyramine-rich foods such as ripe cheese.[5] Their mechanism of action has been extensively investigated and is due to covalent binding to the flavin adenine dinucleotide cofactor at the active site of MAO. Thus, they are suicide inhibitors and MAO-B activity can only be restored by de novo synthesis.[6] The breakdown of the drugs occurs via the loss of the propargyl groups which leads to the formation of *R*-1-aminoindan from rasagiline and *R*-methamphetamine from selegiline (Figure 1).[3,4,7]

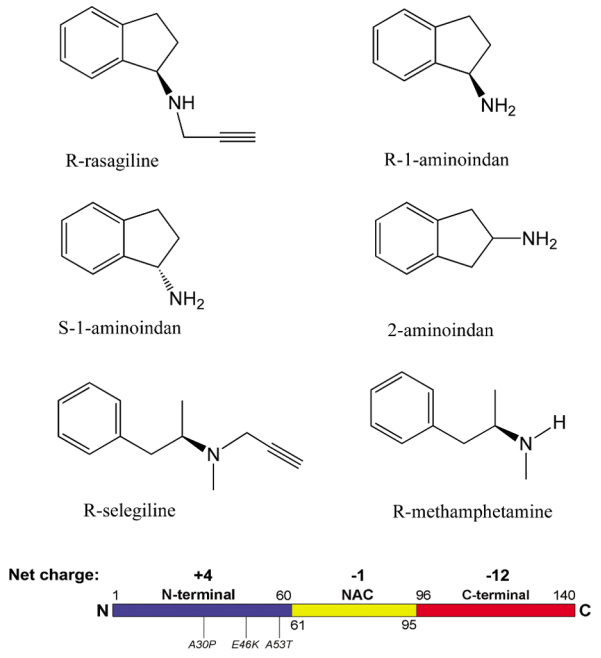


Figure 1. Structures of drugs and the domain structure of AS.

There has been considerable debate about the possible neuroprotective effects of rasagiline and selegiline which could be of great importance clinically since progression of the disease might be slowed.[8−10] Both drugs can prevent the death

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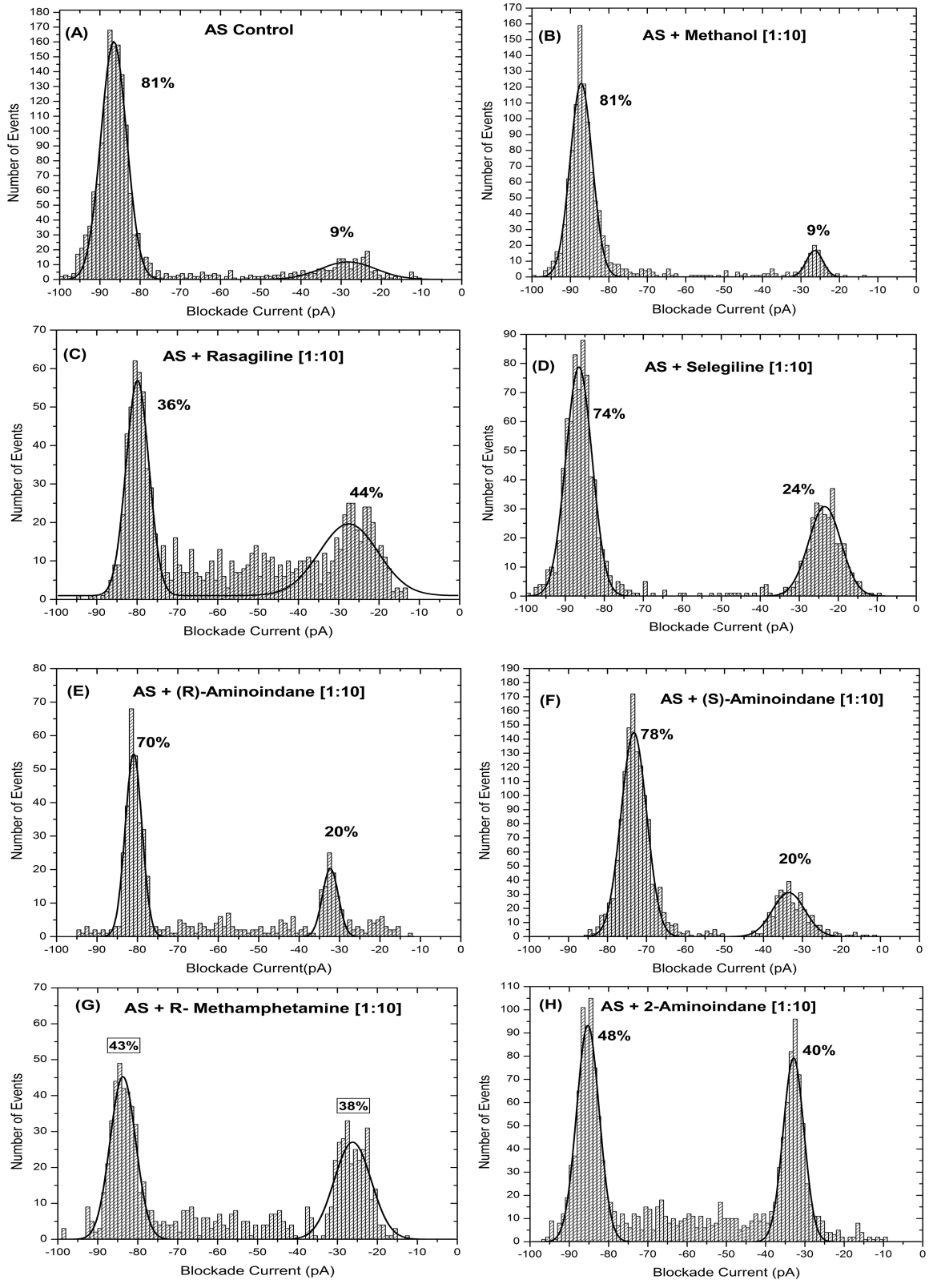

Figure 2. Blockade current histograms for 1 μM AS with 10 μM drug: (A) AS alone, (B) Control AS with methanol (1%), (C) AS with rasagiline, (D) AS with selegiline, (E) AS with *R*-1-aminoindan, (F) AS with *S*-1-aminoindan, (G) AS with *R*-methamphetamine, and (H) AS with 2-aminoindan.

of neuronal cells in culture, but rasagiline is more potent.[11] As well, it would appear that the major metabolite of rasagiline is itself neuroprotective whereas *R*-methamphetamine produced from selegiline is neurotoxic because of sympathomimetic effects.[4,11] Recently, we demonstrated by nanopore analysis that *S*-methamphetamine binds to the intrinsically disordered protein α-synuclein (AS) and forms a complex which is more compact than the native protein.[12] Here we show that both

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rasagiline and *R*-1-aminoindan can bind to AS probably forming a loop structure while, in contrast, selegiline binds and forms a more compact structure similar to both *R*- and *S*-methamphetamine.

The pathobiology of PD is extremely complex and nearly 20 genetic loci which increase the risk for PD have been identified and many of them have been confirmed by linkage analysis.[13] The function of several of the resulting proteins have not been identified but some of them are clearly involved in the clearance of misfolded proteins or damaged mitochondria. For example, the Parkin protein is a ubiquitin ligase which provides a link to the proteasomal clearance system and PINK1 binds to damaged mitochondria and can intitiate mitophagy.[14,15] However, the primary cause of PD appears to be the misfolding of AS and aggregation into Lewy bodies which are found as inclusions upon postmortem examination.[16,17] Although rare, three genetic mutations in the AS gene (*SNCA*) have been discovered which lead to autosomal dominant early onset PD.[18] All of these mutant AS fibrillize more readily than wild type AS and presumably can eventually aggregate into Lewy bodies.[19,20] As well, gene duplication and triplication of *SNCA* is also a risk factor for PD and these results have been replicated in animal models of PD.[21] Perhaps of most significance is that injection of misfolded AS into mouse brain leads to neuronal cell death which can then spread to other sites.[22,23] As well, fetal neurons which were transplanted into the brains of PD patients eventually develop Lewy body pathology, suggestive of cell to cell transmission.[24,25] A possible link between the bewildering array of genetic mutations can be formulated as follows: misfolded AS overwhelms the protein clearance machinery which leads to an increase in reactive oxygen species which, in turn, damages mitochondria.[26,27] Whatever the sequence of events, misfolding of AS clearly plays a central role, and the search for drugs which can prevent this process is imperative.

RESULTS

Nanopore analysis is a method for interrogating the conformation of single molecules as they interact with a pore.[28−32] The pore, in this case α-hemolysin, is embedded in a membrane which separates two chambers and thus a current flows through the pore upon application of a voltage. When a molecule interacts with the pore, an event is recorded which is characterized by the magnitude of the blockade current (I) and the time for which the blockade occurs (T).[33] Native AS (140 amino acids) can readily translocate the pore because it is unfolded and the C-terminus nominally carries twelve negative charges (Figure 1) so that it can be electrophoretically driven through the pore under the influence of an applied positive potential.[34−36] Upon removal of the C-terminus or folding of the protein into a more compact conformation, translocation is prevented and molecules tend to bump into the pore before diffusing away.[12] In general, bumping events have smaller values of I and shorter values of T.[37,38] A third type of event, called intercalation, has also been described in which an unfolded loop or end of a protein enters the lumen of the pore before diffusing back to the *cis* side.[37,38] Intercalation events tend to have intermediate values of both I and T.

Nanopore analysis of AS alone gives rise to blockade current histograms with a major peak at −86 pA comprising about 90% and a small bumping peak at at about −25 pA with about 10% of the events (Figure 2A).[12] This event profile is not affected by the addition of 5−10 μL of methanol (Figure 2B). The peak at −86 pA is due to translocation events because an increase in voltage leads to a decrease in the event time, as expected for a negatively charged protein being driven through the pore.[34] The blockade current profiles after the addition of 10 μM rasagiline, selegiline, *R*- and *S*-1-aminoindan, *R*-methamphetamine, and 2-aminoindan are shown in Figure 2C−H. Upon addition of rasagiline (Figure 2C), the translocation peak of AS decreases in magnitude and shifts to −80 pA and there is a corresponding increase in the proportion of bumping events. Thus, rasagiline binds to AS and causes a significant change in the conformation. There are also a significant number of events with intermediate blockade currents which may represent partially folded or weakly bound structures. In contrast, in the presence of selegiline (Figure 2D), the major peak remains at −87 pA and there is a small increase in the proportion of bumping events. Thus, selegiline only binds weakly to AS. A major metabolite of rasagiline is *R*-1-aminoindan, and it, too, causes a shift in the major peak to about 81 pA (Figure 2E), although there are fewer intermediate blockade events compared to rasagiline. Suprisingly, the other isomer, *S*-1-aminoindan (Figure 2F), also binds to AS, but the major peak is now found at −73 pA, demonstrating that *S*-1-aminoindan binds tightly but causes AS to fold into a different conformation compared to rasagiline or *R*-1-aminoindan. The event profile for *R*-methamphetamine (Figure 2G) is similar to that of selegiline from which it is formed after loss of the propargyl group (Figure 1). 2-Aminoindan, which can be envisioned as a closed-ring form of methamphetamine, also yields a similar event profile to selegiline and *R*-methamphetamine (Figure 2H), although the proportion of bumping events is significantly increased (44% for 2-aminoindan, 38% for *R*-methamphetamine and 25% for selegiline) which is indicative of tighter binding.

The characteristic times of the events for the major peaks for rasagiline, selegiline, and the amnoindans are listed in Table 1.

Table 1. Characteristic Times for the Major Events for AS with and without Drugs at 100 mV unless Stated Otherwise

sample	peak position (pA)	time (ms)[a]
AS	−86	0.49
AS + rasagiline at 75 mV	−80	0.37
AS + rasagiline at 100 mV	−80	0.41
AS + selegiline	−86	0.46
AS + 2-aminoindan	−86	0.46
AS + *R*-1-aminoindan	−80	1.23
AS + *S*-1-aminoindan	−73	0.72
N-terminal AS	−30	0.11
N-terminal AS + rasagiline	−30	0.15

[a]The error is estimated to be ±10%

For rasagiline, the times are shown for two voltages and were calculated to be 0.37 ± 0.4 ms at 75 mV and 0.41 ± 0.4 ms at 100 mV. Since these values are not significantly different, it is not possible to determine whether the AS/rasagiline complex can translocate. In the presence of selegiline or 2-aminoindan at 100 mV, the value of T is 0.46± 0.05 ms which as expected is not significantly different from the value previously reported for AS alone.[34] However, the peak at −80 pA for *R*-1-aminoindan and the peak at −73 pA for *S*-1-aminoindan give characteristic times of 1.23± 0.12 ms and 0.72± 0.07 ms, respectively, both of which are significantly longer than in the absence of drug.

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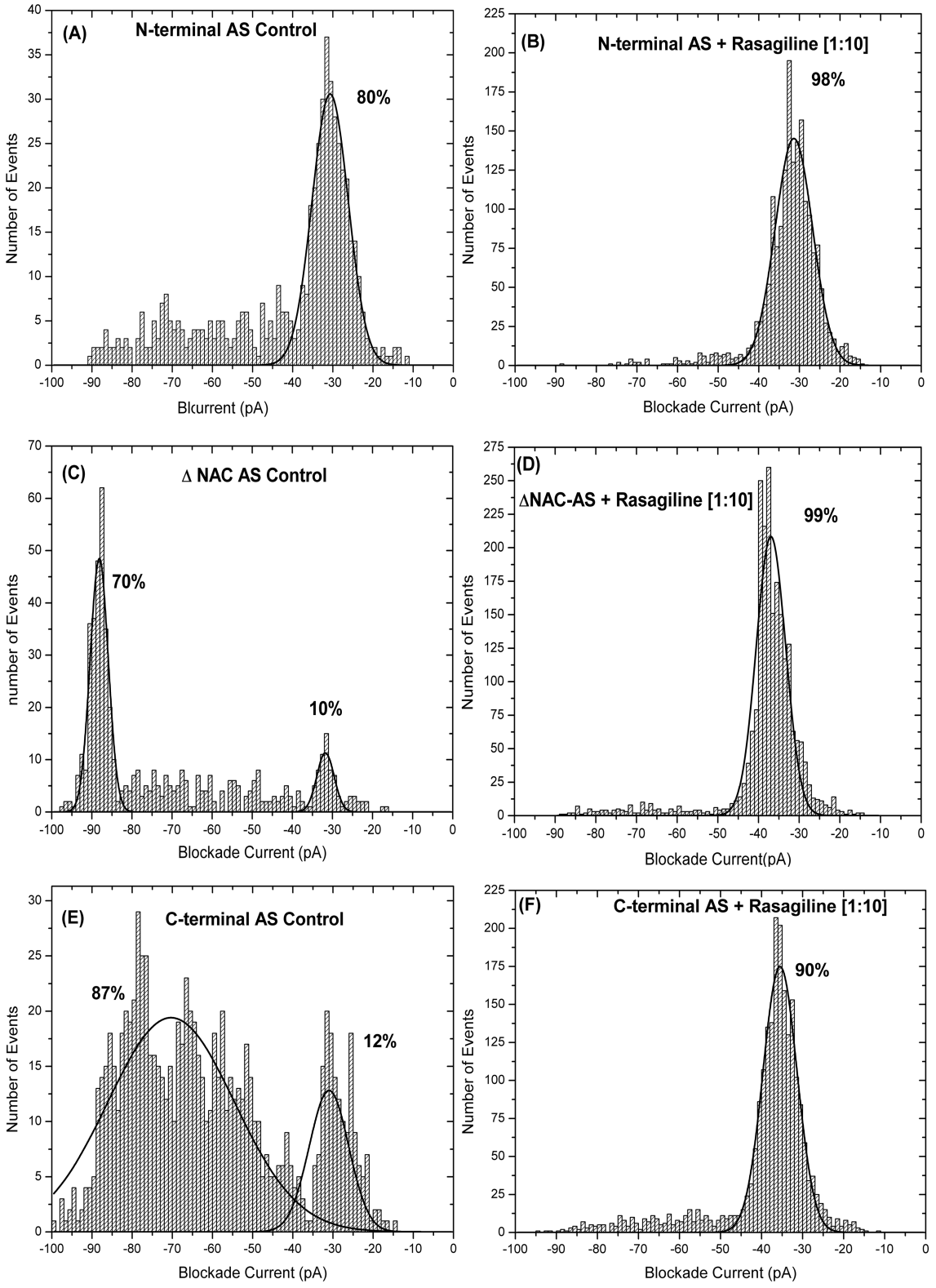

Figure 3. Blockade current histograms for the peptide fragmnets of AS (1 μM) with rasagiline (10 μM): (A) N-terminal AS, (B) N-terminal AS with rasagiline, (C) ΔNAC AS, (D) ΔNAC AS with rasagiline, (E) C-terminal AS, and (F) C-terminal AS with rasagiline.

Further elucidation of the binding site for rasagiline was performed by studying the binding to peptide fragments of AS (Figure 3). Blockade current histograms for the N-terminus of AS (1−60) alone or with rasagiline are shown in Figure 3A,B. As discussed previously,[12] the N-terminal fragment is positively charged and interacts with the pore via diffusion rather than being electrophoretically driven into the pore. Thus, most events are bumping as shown by the major peak at −30 pA. However, there are about 30% of events between −45 and 90 pA, and these are almost completely eliminated when rasagiline is present. As well, there is a significant change in the characteristic time of the bumping events from 0.11 to 0.15 ms

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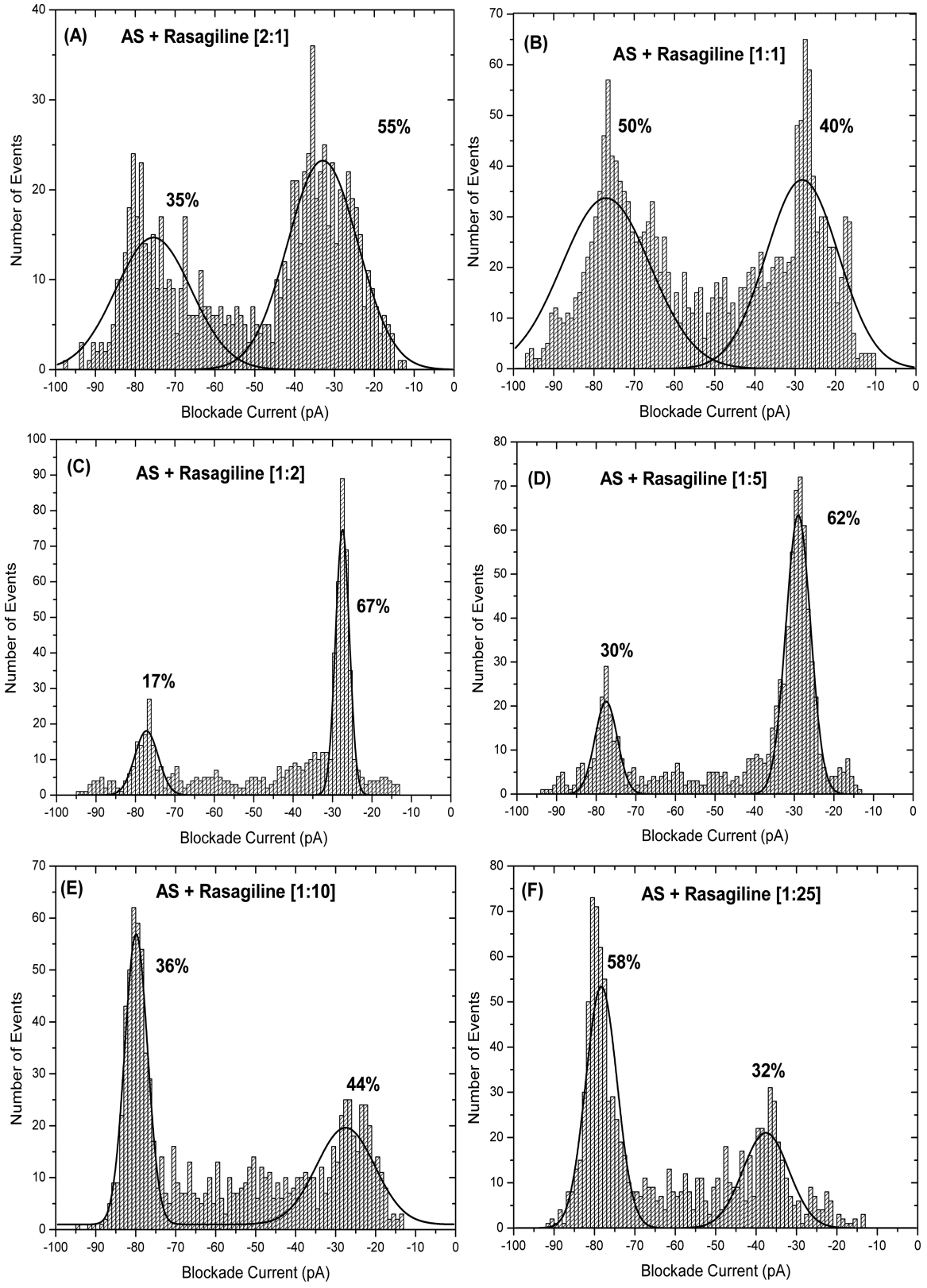

Figure 4. Blockade current histograms for 1 μM AS with increasing concentrations of rasagiline: (A) 0.5 μM rasagiline, (B) 1 μM rasagiline, (C) 2 μM rasagiline, (D) 5 μM rasagiline, (E) 10 μM rasagiline, and (F) 25 μM rasagiline.

(Table 1). For the ΔNAC fragment and the C-terminus (96–140), it is clear (Figure 3D,F) that the presence of rasagiline causes a large increase in the percentage of bumping events. Therefore, it can be concluded that rasagiline binds to both the N- and C-termini of AS and that the NAC region is not required for binding.

The above results suggest that AS may have more than one binding site for rasagiline. Therefore, the effects of increasing

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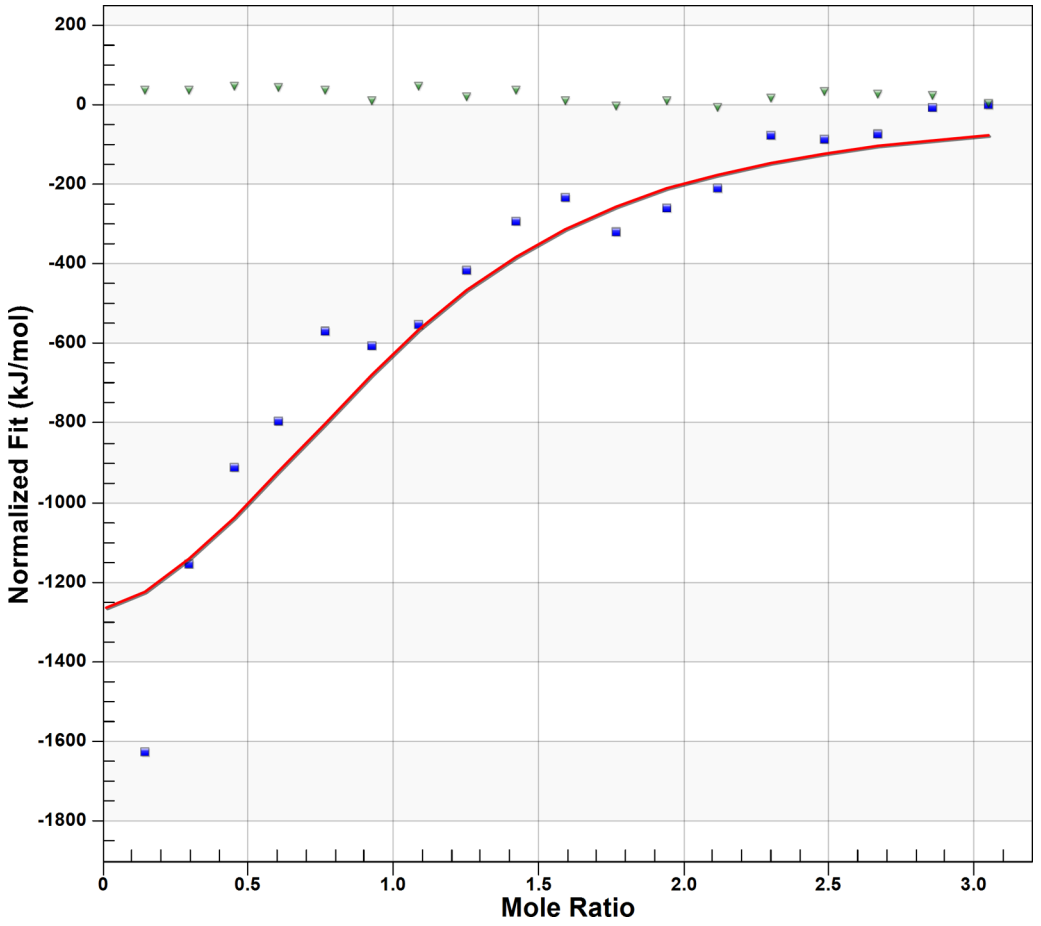

Figure 5. Isothermal titration calorimetry for AS with rasagiline (blue square) and selegiline (green down triangle). The line is the best fit isotherm with $n = 1$ and $K_a = 6.9 \times 10^5$ M^{-1}. The enthalpy changes with selegiline are too small to allow an estimate of the binding parameters.

concentrations of the drug on the blockade current profiles were examined (Figure 4). At 0.5 μM, that is, a ratio of two rasagiline to one AS, there is a significant increase in the bumping peak and the putative translocation peak becomes very broad (Figure 4A). At 1 μM rasagiline, a sharper bumping peak begins to form at about −28 pA (Figure 4B), and at 2 μM the majority of events are found at −28 pA (Figue 4C) with a very narrow distribution which is consistent with a single type of conformation.[39] As well, the translocation peak at both 2 and 5 μM is at about 76 pA. Further increases in the concentration of rasagiline result in a decrease in the bumping peak and an increase in the translocation peak which is now found at −80 pA (Figure 4D−F). At 25 μM rasagiline, the peak at −80 pA represents a majority of the events and the broad bumping peak has shifted to about −38 pA. It can be concluded that several different conformations of AS can occur depending on the concentration of rasagiline.

The binding parameters for rasagiline in the same buffer as for nanopore analysis was measured by isothermal titration calorimetry (ITC) (Figure 5). It was discovered that rasagiline had a very large enthalpy of dilution, and therefore, it was placed into the thermal chamber and AS was injected from the syringe. With the number of binding sites, $n = 1$, K_a was calculated to be $6.9 \times 10^5 \pm 0.3$ M^{-1}. The enthalpy change for selegiline under the same conditions is much smaller, and no meaningful binding constant could be estimated.

DISCUSSION

Epidemiological studies have shown that nicotine consumption lowers the incidence of PD and may be neuroprotective.[40] In contrast, methamphetamine addicts have a higher incidence of PD and the drug or its metabolites are neurotoxic.[41] It is also suspected but not proven that rasagiline is neuroprotective whereas the other common MAO-B inhibitor, selegiline, is not.[4,8] Rasagiline is metabolized to *R*-1-aminoindan, whereas selegiline can be broken down to *R*-methamphetamine.[4,8] Inspection of the structure of these drugs reveals that putative neuroprotectants contain an aryl-carbon-amino moiety compared to aryl-carbon-carbon-amino for those that are neurotoxic. The nanopore results reported here help to clarify these distinctions.

The current blockade profiles (Figure 2) for AS in the presence of rasagiline and *R*-1-aminoindan both show a new peak at −80 pA, whereas for selegiline and *R*-methamphetamine the original AS peak at −86 pA remains and there is an increase in the percentage of bumping events at about −25 pA. An estimate of the binding constant can be made from these profiles.[37] For rasagiline at 10 μM with 1 μM AS, there is at least 90% complex formation because there is no discernible peak at −86 pA (Figure 2C). Thus, $K_a > 0.9/(9.1 \times 0.1) \times 10^6$ or approximately 10^6 M^{-1} which is similar to the value of 6.9×10^5 M^{-1} obtained from ITC (Figure 5). Similarly, for selegiline, the bumping peak increases from about 9% to about 24%, suggesting a maximum of 15% complex formation. Therefore, $K_a < 0.15/(9.85 \times 0.85) \times 10^6$ or approximately 2×10^4 M^{-1}

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which again was verified by ITC. 2-Aminoindan which is also neurotoxic[42] can be considered as a closed ring analogue of methamphetamine and does not yield a blockade current peak at −86 pA. In this case, the bumping peak increases to to about 40%, so K_a for 2-aminoindan may be as high as approximately 5 × 10^4 M^{-1}. Finally, upon binding *S*-1-aminoindan, the blockade current histogram of AS shows a new peak at −73 pA. It is interesting that the *S*-isomer of rasagiline has no MAO-B activity but is still a potent neuroprotectant,[43,44] and thus, the conformation of AS induced by binding *S*-1-aminoindan may also prevent AS from misfolding.

The effect of different concentrations of rasagiline on the blockade current profiles (Figure 4) demonstrates that rasagiline causes AS to adopt a number of different conformations. Even at a 2:1 ratio of AS to drug (Figure 4A), most of the original peak at −86 pA has disappeared, suggesting that 1 rasagiline can bind 2 AS molecules. As well, there is good evidence that rasagiline can bind to sites in both the N- and C-termini but the NAC region is not required. The simplest model to explain these findings is shown in Figure 6.

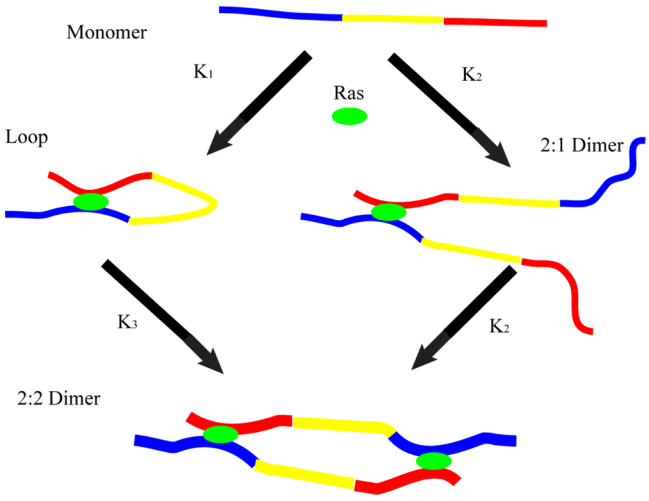

Figure 6. Proposed model for the conformations of AS induced by rasagiline (Ras). The relative concentrations of the loop structure, 2:1 dimer, and 2:2 dimer will depend on the concentrations of AS, rasagiline and the relative values of the binding constants.

The relative concentrations of loop structure, 2:1 dimer, and 2:2 dimer will depend on the concentrations of AS, rasagiline and the relative values of the binding constants. It seems reasonable to suggest that both the loop structure and the 2:1 dimer can translocate by entry of the negatively charged C-terminus into the pore followed by unfolding as the drug is stripped from the protein by the intense electric field at the entrance to the pore.[45] However, the 2:1 dimer is an extended structure and therefore is more likely to give rise to bumping events. At higher concentrations of rasagiline, the molecules may adopt a 2:2 dimer. The 2:2 dimer is more compact and therefore is more likely to translocate than the 2:1 dimer. In support of this explanation, it was observed that the proportion of translocation events increases as the concentration of rasagiline increases (Figure 4C−F).

There is good evidence that the fibrils and aggregates of AS contain β-sheet structures, although it is not clear whether they are parallel or antiparallel sheets. For example, the initial AS oligomers may adopt an antiparallel structure whereas the fibrils are mostly parallel β-sheets.[46−48] The β-rich core extends from within the N-terminus at residue 38 to the end of the NAC region at residue 95, suggesting that formation of the loop or dimer structures of Figure 6 will inhibit the formation of β-sheet structures. Therefore, molecules such as rasagiline, caffeine, nicotine, curcumin, and dopamine[35,36] which can form such structures may prevent aggregation of AS, whereas others such as selegiline and methamphetamine which only bind to the N-terminus do not.[12] Nanopore analysis will prove useful in distinguishing between the two types of interaction and in the search for effective treatments for PD.

METHODS

Nanopore Analysis. The buffer contained 1 M KCl and 10 mM Hepes/KOH (pH 7.8), and the method has been described previously.[33−39] Briefly, a lipid bilayer was made by painting 2-dipheytanoyl-*sn*-glycero-3-phosphocholine from Avanti Polar Lipids (Alabaster, AL) onto an aperture in a Teflon perfusion cup. The cup separated the *cis* and *trans* compartments with volumes of 1 mL. An applied potential of −100 mV was generated using a Dididata 1440A system (Axon Instruments). The bilayer was thinned until the thickness resulted in a capacitance between 65 and 75 pF. α-Hemolysin from Sigma-Aldrich Canada, Ltd. (Oakville, Ontario) was diluted to 1 μg/mL, and 5 μL aliquots were added to the *cis* side until a pore was inserted giving a current of 98−100 pA. Once stable, AS from rPeptide (Bogart, GA) was added to the *cis* side to a final concentration of 1 μM. Stock solutions of drugs (from Sigma-Aldrich) were dissolved in methanol and diluted into the *cis* compartment to a maximum concentration of 1% methanol. The event profiles were obtained, and blockade currents were plotted with a Gaussian function using Clampfit (Axon Instruments) and Origin 7. Blockade times were obtained by fitting an event time histogram to a single exponential.

Isothermal Titration Calorimetry. ITC was performed in a Nano-ITC calorimeter (TA Instruments, New Castle, PA) which has a volume of 250 μL. Samples (50 μM AS in the syringe and 5 μM drug in the thermal chamber) were made by dilution in nanopore buffer, 1 M KCl, and 10 mM Hepes/KOH (pH 7.8). The samples were degassed in a vacuum for 1.5 h. Experiments were performed at 20 °C at a stir rate of 250 rpm. There were 21 injections at 250 s intervals. The majority of injections had a volume of 2.5 μL with the exception of the first two (injection 1 0.18 μL, injection 2 2.26 μL) which were omitted from the results. A control was performed with AS in the syringe and buffer in the compartment. The control signal was subtracted from the results with drug. The data was then fitted using NanoAnalyze (TA Instruments).

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Author Contributions

J.K. and O.T. performed the experiments with the supervision of J.S.L. who wrote most of the paper.

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Notes

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