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Active Site Mutations Change the Cleavage Specificity of Neprilysin

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Abstract

Neprilysin (NEP), a member of the M13 subgroup of the zinc-dependent endopeptidase family is a membrane bound peptidase capable of cleaving a variety of physiological peptides. We have generated a series of neprilysin variants containing mutations at either one of two active site residues, Phe¹⁶³ and Ser¹⁶⁶. Among the mutants studied in detail we observed changes in their activity towards leucine⁵-enkephalin, insulin B chain, and amyloid β₁₋₄₀. For example, NEP⁵⁶³L displayed an increase in preference towards cleaving leucine⁵-enkephalin relative to insulin B chain, while mutant NEP⁵⁵⁶⁴E was less discriminating than neprilysin. Mutants NEP⁵⁶³L and NEP⁵⁵⁶⁴E exhibit different cleavage site preferences than neprilysin with insulin B chain and amyloid β₁₋₄₀ as substrates. These data indicate that it is possible to alter the cleavage site specificity of neprilysin opening the way for the development of substrate specific or substrate exclusive forms of the enzyme with enhanced therapeutic potential.

Introduction

Neprilysin (NEP), also known as neutral endopeptidase 24.11, CD10, enkephalinase, and CALLA, is a member of the M13 subgroup of zinc-dependent endopeptidases [1]. NEP was originally discovered in rabbit kidney as a peptidase that cleaves insulin B chain [2]. Subsequent studies showed that NEP is widely expressed throughout mammalian tissues, including the lung, male genital tract, fibroblasts, various epithelia, and at neural synapses in the central nervous system [3–5]. The enzyme cleaves a variety of physiological substrates including bombesin-like peptides, amyloid β peptides (Aβ), leucine⁵ or methionine⁵-enkephalin, bradykinin, atrial natriuretic factor (ANF), and substance P [6–9]. NEP exhibits a preference for cleavage on the amino terminal side of hydrophobic residues [10].

Because of its multiple targets, NEP has been the focus of numerous studies attempting to modulate its activity for therapeutic purposes. One such target is the use of NEP to reduce Aβ peptide levels in Alzheimer’s disease, since the oligomerization of Aβ has been linked to the etiology of this disease [11]. Indeed, in studies with transgenic mice NEP expression decreases the level of Aβ [12–15] and ameliorates cognitive deficits typically attributed to AD [16]. In yet another application inhibitors of NEP were developed to block its “enkephalinase” activity to increase the concentration of enkephalins in the brain and thus their analgesic effect [17]. Peripherally expressed NEP may have a role in appetite control and obesity. NEP deficient mice become obese [18], while a peripherally administered NEP inhibitor that does not cross the blood-brain barrier increased food intake and subsequently led to obesity. Recently, an NEP inhibitor was shown to increase female genitalia blood flow in rabbits by preventing vasoactive intestinal peptide (VIP) cleavage [19]. This could potentially lead to the use of NEP as a therapeutic agent in the treatment of female sexual arousal disorder.

While methods to modulate NEP activity have displayed the potential for therapeutic use, they also reveal a paradox to their usage. For example, using NEP to lower Aβ may indeed decrease the amount of the target substrate; it may also have undesired consequences by removing other physiologically important products such as the enkephalins or vasopressin. Alternatively, inhibiting NEP to enhance opioid levels will likely cause an increase in Aβ, which would result in an increased risk in the development of Alzheimer’s disease.

A strategy to bypass the potential problems associated with the substrate promiscuity of NEP is to alter its specificity towards a target substrate thus reducing potential off-target effects. There is ample precedence to apply such a strategy. For example, substitutions within the active site of trypsin, although decreasing activity, shifted the relative preference for arginine versus lysine [20]. Similarly, a series of mutations in Rous sarcoma virus protease displayed altered amino acid preferences at particular substrate positions, allowing position-by-position control of substrate specificity [21]. Using thermolysin as a homology model, we were able to show that conversion of Val¹⁷² to Leu produced a form of NEP which reacted with substrates with small Pⁱ residues essentially the same as wild-type enzyme, yet substrates containing bulky Pⁱ residues exhibited a decreased Vmax with little change in Km [22]. This study, although limited in scope, demonstrated the

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feasibility of altering NEP substrate specificity. The nomenclature of Schecter and Berger (Schechter I, Berger A. (1968) Biochem. Biophys. Res. Commun. 32: 890-902) is used where residues of the substrate C-terminal to the site of cleavage are designated P1', P2', P3', etc as they move away from the scissile bond and residues N-terminal to the scissile bond are designated P1, P2, P3, etc as they move away from the scissile bond. The corresponding binding sites on the enzyme are designated S1', S2', S3', and S1, S2, S3, etc respectively.

By analyzing the crystal structure of NEP in complex with the inhibitor phosphoramidon [23], we have initiated a rational design approach to mutate NEP active site targeting residues likely to interact with substrates. In this study, we explore NEP substrate specificity by generating NEP mutant libraries of two active site residues, Phe563 which is part of the S1' binding site and Ser546 which appears to contribute to the S2/S3 binding site. A number of these mutants displayed differential changes in activity toward physiological substrates including changes in cleavage site preferences. Together, these data support the hypothesis that amino acid changes in the active site of NEP can potentially give rise to therapeutically relevant forms of NEP.

Results and Discussion

Selection of sites for mutagenesis

Mutations were made at the Phe563 and Ser546 sites in a secreted form of human NEP (shNEP) expressed as a C-terminal hexahistidine fusion protein. The NEP crystal structure reveals that Phe563 forms part of the S1' substrate binding pocket believed to impart the preference for hydrophobic/aromatic P1' residues at this position. Figure S1. Phe563 is located in a coil region just prior to the helix containing the active site residues. Ser546 is part of a β-sheet lining the substrate-binding site [23] and is positioned to interact with the P2 or P3 residues of a bound substrate on the carboxyl side of the scissile bond. Based on the NEP crystal structure, the position of both Phe563 and Ser546, and their conservation among species, we hypothesized that these residues contribute to substrate specificity.

Expression of mutant NEP

To test the contribution of Phe563 and Ser546 to catalysis we used degenerate oligonucleotides to construct NEP libraries in which we introduced amino acid substitutions at these positions. Substitutions made at Phe563 included valine, leucine, methionine, isoleucine, serine, histidine, aspartic acid, arginine, glutamine, asparagine, and lysine. Substitutions made at Ser546 included asparagine, and lysine. Substitutions made at Ser 546 included isoleucine, serine, histidine, aspartic acid, arginine, glutamine, and lysine. Substitutions made at Ser 546 included isoleucine, serine, histidine, aspartic acid, arginine, glutamine, and lysine. Individual mutant library members were transfected in HEK293 cells and analyzed for expression. Of the seventeen sequences examined, five mutants, NEPF563L, NEPF563V, NEPF563M, NEPF563L, and NEPS546E, expressed at levels near to that of wild-type NEP and were selected for further purification and analysis. The low expression of other mutants appeared to be due to their cellular instability as they all produced similar amounts of mRNA.

Figure S2, which did not correlate with protein expression nor did the poorly expressing mutants accumulate intracellularly. These results suggest that active-site residues Phe563 and Ser546 play a role in overall protein folding and/or stability and that the non-expressing mutants were likely degraded intracellularly.

The five expressing NEP mutants were purified by nickel affinity chromatography, and the amount of NEP present determined by Sypro ruby staining of SDS-PAGE gels. We initially compared Sypro Ruby and Western blot analysis for enzyme quantitation and obtained equivalent results with either method, Figure S3.

Reaction of NEP mutants with the synthetic substrate Glut-Ala-Ala-Phe-MNA

Activity assays were first performed using the synthetic peptide Glut-Ala-Ala-Phe-MNA. This substrate is cleaved between the Ala-Phe peptide bond and thus any effects of mutations on the cleavage at this site will be reflected in the reaction kinetics. We demonstrated that Glut-Ala-Ala-Phe-MNA hydrolysis by NEP and each of the studied mutants was completely inhibited by the relatively specific inhibitor, phosphoramidon at 100 µM, and the highly specific inhibitor CGS 24592 [24] at 10 nM, thus demonstrating that hydrolysis was attributed to NEP or its variant and not a contaminating protein.

Kinetic constants for mutants determined with Glut-Ala-Ala-Phe-MNA as substrate are presented in Table 1. These kinetic constants were derived under first-order assay conditions monitored in a continuous mode. The wild-type enzyme and the NEP563L mutant exhibited essentially the same specific activity of 46 and 44 pmoles/min/ng, respectively, while the NEP563I, NEP563V, NEP563M, and NEP563L mutant activities varied from ~25% to 45% of the wild-type enzyme. Table 1. Kₚ values varied ~2.5 fold ranging from 51 to 118 µM, with Vmax/Km values varying three fold or less. Thus mutating Phe563 and Ser546 produce small but detectable affects on the cleavage of Glut-Ala-Ala-Phe-MNA confirming that these residues contribute to catalysis.

**Table 1.** The specific activity towards glutaryl-Ala-Ala-Phe-MNA cleavage is reduced in NEP mutants.

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>Kₚ</th>
<th>Kₘ insulin B chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pmoles/min/ng)</td>
<td>(µM)</td>
<td>(µM)</td>
</tr>
<tr>
<td>NEP</td>
<td>45.9±2.5</td>
<td>51±11</td>
</tr>
<tr>
<td>NEP563L</td>
<td>17.7±0.6</td>
<td>83±4</td>
</tr>
<tr>
<td>NEP563V</td>
<td>43.6±0.1</td>
<td>81±11</td>
</tr>
<tr>
<td>NEP563M</td>
<td>19.2±0.4</td>
<td>87±8</td>
</tr>
<tr>
<td>NEP563I</td>
<td>11.2±0.3</td>
<td>51±10</td>
</tr>
<tr>
<td>NEP563E</td>
<td>20.1±0.6</td>
<td>74±10</td>
</tr>
<tr>
<td>NEP563N</td>
<td>ND*</td>
<td>118±7</td>
</tr>
<tr>
<td>NEP546E</td>
<td>ND*</td>
<td>73±6</td>
</tr>
</tbody>
</table>

All assays were conducted at 37°C at in 20 mM MES buffer, pH 6.5. ND = not determined.

*The concentrations of NEP556E and NEP5546E were too low to quantify, however, they were active enough to determine Kₚ.

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The specific activities of the various mutants with the physiological substrate leu-ENK showed a similar pattern as observed with Glut-Ala-Ala-Phe-MNA. NEPF563L had near wild-type levels of activity for both Glut-Ala-Ala-Phe-MNA and leu-ENK, while NEPF563M, NEPF563I, and NEPS546E all showed approximately 40–60% activity towards these substrates, and NEPF563V exhibited a 75–85% decrease in activity towards both. Thus the effects of these mutations can be attributed to those produced for cleaving N-terminal to single phenylalanyl residue.

Insulin B chain and Ab1−40 contain multiple cleavage sites. Cleavage at any one of these sites will result in peptide disappearance as determined by HPLC. Compared to wild-type NEP, mutants NEPF563V, NEPS546E, NEPF563I, and NEPF563M all exhibited reduced hydrolysis rates for insulin B chain (p values of 0.03 or lower), whereas with NEPF563I the hydrolysis rate was higher (p = 0.03). With Ab1−40 as substrate all of the mutants showed reduced rates of hydrolysis (all exhibited p values < 0.05).

When the relative cleavage rates for insulin B chain, Ab1−40, and leu-ENK were compared between NEP and the various mutants there was a discernable change in substrate preference. For example, NEPF563L hydrolyzed insulin B chain at a rate 1.4 times faster than NEP, but cleaved Ab1−40 at nearly half the rate of the wild-type enzyme. Thus NEPF563L exhibits a greater than 2 fold preference for insulin B chain over Ab1−40. NEPF563V cleaved leu-ENK at 1/6 the rate of the wild-type enzyme and Ab1−40 at 1/12 the wild type rate, Table 2, increasing the preference of this mutant for leu-ENK.

To further demonstrate that the NEP mutants differ in their ability to discriminate between substrates, we measured rates of cleavage of a mixture of two substrates, leu-ENK and insulin B chain. The rate of cleavage of a substrate mixture is determined by both the kcat for that substrate as well as the affinity for the substrate. As can be seen in Table 3 all NEP mutants showed a decrease in the rate of hydrolysis of both leu-ENK and insulin B chain when present together. This is expected, given that each peptide acts as a competitive inhibitor of the other. NEPF563L, NEPF563I, and NEPF563M all showed approximately the same wild-type enzyme ratio of rates for leu-ENK/insulin B chain indicating no change in overall substrate preference. However, NEPF563V exhibited a shift in substrate preference toward leu-ENK, while the substrate preference for NEPS546E shifted more toward insulin B chain, even though the rate remained higher for leu-ENK. That is the ratio for leu-ENK/insulin B chain cleavage is 3 for NEPS546E compared to 6 for wild-type NEP. Thus these single amino acid substitutions had a measurable effect on substrate preference.

### Identification of NEP cleavage sites in insulin B chain

We next looked in more detail on the effect of the NEPF563 and NEPS546 mutations on the hydrolysis rates at individual cleavage sites in insulin B chain. We first tested for a change in the affinity of insulin B chain for mutant NEPs by using insulin B chain as an alternate substrate (competitive) inhibitor of Glut-Ala-Ala-Phe-MNA hydrolysis. We found that there was no dramatic change in the Kc for any of the mutants, with variations of two fold or less (Table 1).

Degradation of insulin B chain by wild-type NEP was analyzed by reverse-phase HPLC following the appearance of each product as a function of time, Figure 1. Product peaks were collected and subsequently analyzed by mass spectrometry to determine their identities. As shown in Table 4 and the insert in Figure 1 this analysis identified seven cleavage sites. Based on the order of appearance of each peak, it is likely that insulin B chain 1–10, insulin B chain 11–17, insulin B chain 17–24 and its partner peak insulin B chain 15–30, insulin B chain 16–23, and insulin B chain 24–30 are all products of primary cleavages. These products appear at the first time point when approximately 15% hydrolysis of insulin B chain had occurred (30 minutes). At 30% hydrolysis (90 min.), peaks corresponding to insulin B chain 1–16 and insulin B chain 16–23 become apparent at ≈30% insulin B chain hydrolysis. It should be noted that the expected product peaks insulin B chain 11–27 and insulin B chain 12–30, the partner products of insulin B chain 10 and insulin B chain 11, respectively, were identified by mass spectrometry, however the peaks never accumulated significantly above the baseline throughout the incubation. It is likely that these are transient products that are subsequently cleaved contributing to other product peaks (i.e. insulin B chain 24–30).

### Analysis of NEP mutant cleavage of insulin B chain

As a representative of the NEPF563 and NEPS546 mutants, we compared the cleavage profile of NEPF563V and NEPS546E to that of NEP using time course experiments, Figure 2. By adjusting the amount of NEP mutant used, the rate of hydrolysis of insulin B chain by all NEP forms was virtually identical. The overall...
cleavage profile at 30% substrate hydrolysis revealed that all of the product peaks observed with NEP are present with the mutant enzymes indicating that there were no unique or missing cleavage sites between NEPF563L, NEPS546E and NEP, Figure 2A. Since rates were based on peak areas measured at 214 nm, which in turn is dependent on both the number of peptide bonds and the number of aromatic residues within a given peptide, only the observed rates of change for a particular peptide product can be compared between enzyme forms. A comparison of the rate of change of different peaks within the same enzyme form or between enzyme forms is not valid under our conditions of analysis.

NEP mutations affect cleavage sites preferences in insulin B chain

Table 5 and Figure 2B,C show the rates of product accumulation normalized to the amount of NEP protein present. Relative to wild-type NEP the overall rate of hydrolysis of insulin B chain is slightly increased in NEPF563L and slightly decreased in NEPS546E, Table 2. Thus one scenario is that all sites in insulin B chain would be cleaved at the same relative rate compared to wild-type NEP. Alternatively, the introduced mutations may differentially affect specific cleavage sites. The data in Table 5 clearly shows the latter scenario with differential effects of mutations on specific cleavages. NEPS546E cleaves insulin B chain at an overall rate 0.7 times that of NEP, however it is clear that cleavage at A14-L15 is nearly identical between NEP and this mutant. Cleavage at H5-L6 is well below the overall insulin B chain rate of 0.7 (Figure 2B), while cleavages at H10-L11, Y16-L17, and G23-F24 are all slightly slower than the expected 0.7 times the wild-type rate.

NEPF563L cleaves insulin B chain at a rate 1.4 times that of NEP. Similar to that seen with NEPS546E, NEPF563L products produced from single cleavage sites exhibit noticeably different rates compared to NEP, Figure 2C. Cleavage at A14-L15, H5-L6, and G23-F24 are close to the expected 1.4 times faster that of NEP, but cleavage at H10-L11, L11-V12, and Y16-L17 are slower than NEP, (0.8 times, 0.2 times, and ~0.7 times the NEP rate respectively, rather than the overall 1.4 times faster than the NEP rate).

Based on the data in Table 5 the elevated activity of NEPF563L towards insulin B chain can likely be attributed to an increased rate of cleavage at the primary cleavage site A14-L15. Although this cleavage involves a leucine residue, the finding that cleavage at Y16-L17 is slower than with wild-type NEP shows the enhanced cleavage at A14-L15 is not due to simply the F563L mutation.

**Table 4. Products of insulin B chain hydrolysis by NEP.**

<table>
<thead>
<tr>
<th>Retention Time (min.)</th>
<th>Insulin B Chain Fragment</th>
<th>Expected Mass</th>
<th>Observed Mass</th>
<th>Cleavage Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1</td>
<td>1–5</td>
<td>643.31</td>
<td>643.24</td>
<td>H5-L6</td>
</tr>
<tr>
<td>18.3</td>
<td>1–10</td>
<td>1188.48</td>
<td>1188.39</td>
<td>H10-L11</td>
</tr>
<tr>
<td>22.7</td>
<td>17–24</td>
<td>927.42</td>
<td>927.35</td>
<td>Y16-L17</td>
</tr>
<tr>
<td>24.3</td>
<td>1–11</td>
<td>1301.6</td>
<td>1301.47</td>
<td>L11-V12</td>
</tr>
<tr>
<td>24.7</td>
<td>15–23</td>
<td>1056.51</td>
<td>1056.42</td>
<td>A14-L15</td>
</tr>
<tr>
<td>25.4</td>
<td>1–14</td>
<td>1600.75</td>
<td>1600.64</td>
<td>G23-F24</td>
</tr>
<tr>
<td>26.2</td>
<td>24–30</td>
<td>872.44</td>
<td>872.39</td>
<td>G23-F24</td>
</tr>
<tr>
<td>29.1</td>
<td>17–30</td>
<td>1634.79</td>
<td>1634.64</td>
<td>Y16-L17</td>
</tr>
<tr>
<td>31.9</td>
<td>1–16</td>
<td>1876.89</td>
<td>1876.75</td>
<td>Y16-L17</td>
</tr>
<tr>
<td>33.3</td>
<td>15–30</td>
<td>1910.93</td>
<td>1910.8</td>
<td>A14-L15</td>
</tr>
<tr>
<td>33.6</td>
<td>12–30</td>
<td>2210.87</td>
<td>2209.93</td>
<td>L11-V12</td>
</tr>
<tr>
<td>35.5</td>
<td>11–30</td>
<td>2323.17</td>
<td>2323.06</td>
<td>H10-L11</td>
</tr>
<tr>
<td>36.6</td>
<td>1–30</td>
<td>3493.67</td>
<td>3493.52</td>
<td>(insulin B chain)</td>
</tr>
</tbody>
</table>

NEP mediated hydrolysis was carried out as described in Table 2. The reaction was stopped by adding 10 μL of 5% TFA when approximately half of the substrate had been hydrolyzed (180 min). The acidified reaction mixture was subjected to HPLC as described in figure 1, individual peaks were collected and identified by mass spectral analysis.

doi:10.1371/journal.pone.0032343.t004

Figure 1. Time course for NEP mediated hydrolysis of insulin B chain. Time course assays were conducted by incubation of NEP with 15 μM insulin B chain in 20 mM MES buffer, pH 6.5, at 37°C. A 100 μL aliquot was removed at each time point and 10 μL of 5% trifluoroacetic acid (TFA) was added to stop further hydrolysis. 95 μL were injected into a Vydac C4 column and developed as described in Materials and Methods. Each product was isolated and subjected to mass spectral analysis. Numbers under each peak indicate the identification of the peptide by sequence. Peaks without numbers were not identified.
producing enhanced reactivity toward leucine, but more likely an effect of neighboring residues.

**Identification of NEP cleavage sites in Aβ1–40**

To further study the effect of mutations on cleavage site specificity, time course assays were also performed for the hydrolysis of the physiological substrate Aβ1–40. Like the analysis of insulin B chain, a time course assay was first done with NEP to identify cleavage products, **Figure 3 and Table 6**. After 15% hydrolysis of Aβ1–40 (150 min.) by NEP there were six discernable product peaks corresponding to Aβ1–16, Aβ1–17, Aβ10–17, Aβ20–28, Aβ20–29, and Aβ20–30. At 25% hydrolysis (240 min.), peaks corresponding to Aβ1–9, Aβ4–16, and Aβ4–17 were observed, while at 40% hydrolysis peaks Aβ4–9 and Aβ10–16 appeared. Peak Aβ12–17 was the last peak to be observed at 45% hydrolysis of Aβ1–40 (360 min.). Missing from the HPLC analysis were the C-terminal products resulting from the cleavages at K28–G29, G29–A30, and A30–I31. These products are derived from the trans-membrane region of the amyloid precursor protein (APP) from which Aβ is formed and are rather hydrophobic. It is likely that these peptides were not eluted in the gradient we employed.

Two other studies have identified NEP cleavage sites within Aβ1–40 [25,26]. These studies as well as the current study all detected cleavages at G9–Y10, F19–F20, and A30–I31. Cleavage at A3–F4 was detected in this study as well as by Howell et al. [25,26]. Cleavages at K20–G29 and G29–A30 were detected in this study as well as by Leissring et al. [25,26]. In the current study additional unreported cleavage sites at E11–V12, K16–L17, and L17–V18 were detected. These were previously pointed out as potential cleavage sites, but were not observed [25,26]. Both Leissring and Howell identified cleavage at G33–L34, yet this cleavage was not found in this study. These differences likely reflect differences in the resolution of the Aβ1–40 cleavage products on the HPLC columns used and gradient conditions.

**Analysis of specific cleavage sites in Aβ1–40**

We next compared the Aβ1–40 cleavage profile of the NEPF563L and NEPS546E mutants to that of wild-type NEP, **Figure 4**. Since NEP, NEPF563L, and NEPS546E cleave Aβ1–40 at different rates, the amount of the mutant enzymes used in the reaction was as before adjusted in order to analyze products formed at the same fraction of degradation. Interestingly, although all wild-type peaks were present in the NEPS546E mutant, the peak corresponding to Aβ12–17 was not present among the hydrolysis products of the NEPF563L mutant.

Similar to the analysis conducted with insulin B chain, the linear rate of product accumulation was determined for each peak and normalized to the amount of protein in the reaction, **Table 7**. Unlike the hydrolysis of insulin B chain, the hydrolysis of Aβ1–40, even at the earliest time points, produced products resulting from...
multiple cleavage events. Out of the 12 identified product peaks, only 3 could be produced by a single cleavage. Rates for all cleavages were calculated and are given in Figure 4, B–D, but only the three putative primary cleavage sites can be compared. NEPS546E hydrolyzes Aβ1–40 at an overall rate 0.5 times that of wild-type NEP. Two of the three products resulting from a possible single cleavage by NEPS546E exhibit a higher than predicted rate of cleavage. Cleavage at K16-L17 producing Aβ1–16 is identical rather than half the wild-type rate while cleavage at L17-V18 producing Aβ1–17 was approximately 70% rather than 50% of the NEP rate. The remaining single cleavage site at G9-Y10 shows a slower rate being about 30% that of wild-type enzyme. Thus it would appear that all three of these cleavages contribute to the overall rate and together produce an average rate 0.5 times that of NEP.

Cleavage at L17-V18 is approximately three times faster for NEPS563L compared to NEP. Thus cleavage at L17-V18 for mutant NEPS563L is 70% of the wild-type NEP whereas cleavage at this bond for the NEP563L is 25% of NEP. In order to account for this finding there must either be an undetected cleavage that is significantly reduced in NEPS546E or more likely that NEPF563L exhibits a unique cleavage pattern that yields these products.

The rate of appearance of Aβ1–16 is linear over the entire 360-minute time course for all three enzymes. Aβ1–17, on the other hand, shows a linear increase with both NEPF563L and NEPS546E, but is non-linear with wild-type NEP showing very little increase after 150 min. This is consistent with Aβ1–17 being further metabolized by NEP, most likely by being cleaved at G9-Y10 giving rise to Aβ1–9 and Aβ10–17, both of which show higher peak areas in NEP than with either mutant. In contrast the absence of an obvious reduction of Aβ1–9 and Aβ10–17 in the reaction of NEPF563L suggests these mutants cleave the G9-Y10 bond at a much slower rate.

Although NEPF563L did not produce a discrete Aβ1–9 peak, it appears to cleave the G9-Y10 bond as evidenced by the presence of the products Aβ1–9 and Aβ10–17. Since Aβ1–9 is absent in the NEP563L profile but Aβ10–17 is observed, the cleavage by NEPs563E at G9-Y10 is likely dependent on the cleavage at A3-F4. Aβ1–9 is not further degraded by hydrolysis at the A3-F4 site with both NEP and NEPS546E as evidenced by its linear increase as a function of time. If hydrolysis occurred at the A3-F4 bond of the Aβ1–9 product, one would expect either no time dependent increase or a decrease in the Aβ1–9 peak.

The finding that most of the observed products of Aβ1–40 cleavage result from multiple cleavages, even for early time points, would suggest that NEP is processive in its cleavage of Aβ1–40 and can make several cleavages before product release. Whether the

<table>
<thead>
<tr>
<th>Table 5. Relative rates of accumulation of peaks generated by the NEP and NEP mutant dependent hydrolysis of insulin B chain.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Cleavage</td>
</tr>
<tr>
<td>Cleavage Site</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>H5-L5</td>
</tr>
<tr>
<td>H10-L11</td>
</tr>
<tr>
<td>L11-V12</td>
</tr>
<tr>
<td>A14-L15</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Y16-L17</td>
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<tr>
<td></td>
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<tr>
<td>G21-L24</td>
</tr>
<tr>
<td>Two Cleavages</td>
</tr>
<tr>
<td>Cleavage Sites</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>A14-L15, G21-L24</td>
</tr>
<tr>
<td>Y16-L17, F24-L25</td>
</tr>
</tbody>
</table>

Time course assays were carried out by incubation of NEP with insulin B chain using conditions as described in Table 2. At 0, 30, 60, 90, 120, and 180 min., aliquots of 100 μL were removed followed by the addition of 10 μL of 5% TFA to stop further hydrolysis. Each reaction mixture was subjected to HPLC analysis as in Table 4 and peak areas measured. The rate of accumulation for each peak was calculated from the linear phase of the reaction. doi:10.1371/journal.pone.0032343.t005

![Figure 3. Time course of NEP mediated hydrolysis of Aβ1–40.](figure3.png) Time course measurements were carried out by incubation of NEP with 24 μM Aβ1–40 in 20 mM MES, pH 6.5, at 37°C. 100 μL aliquots were removed at each time point and 10 μL of 5% TFA was added to stop the reaction. Samples were analyzed as described in Figure 1. Numbers under each peak indicate the identification of the peptide by sequence. doi:10.1371/journal.pone.0032343.g003
Table 6. Identification of products resulting from the hydrolysis of Aβ1–40 by NEP.

<table>
<thead>
<tr>
<th>Retention Time (min.)</th>
<th>Amyloid β peptide fragment</th>
<th>Expected Mass</th>
<th>Observed Mass</th>
<th>Cleavage Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>4–9</td>
<td>717.32</td>
<td>717.30</td>
<td>E2\textsuperscript{\textasciitilde}F\textsuperscript{\textasciitilde}G\textsuperscript{\textasciitilde}V\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>10.3</td>
<td>10–16</td>
<td>939.46</td>
<td>939.44</td>
<td>G\textsuperscript{\textasciitilde}K\textsuperscript{\textasciitilde} \textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>14.7</td>
<td>1–9</td>
<td>1032.43</td>
<td>1032.43</td>
<td>G\textsuperscript{\textasciitilde}Y\textsuperscript{\textasciitilde}K\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>15.8</td>
<td>12–17</td>
<td>760.43</td>
<td>760.44</td>
<td>E\textsuperscript{\textasciitilde}I\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}V\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>17.2</td>
<td>20–28</td>
<td>964.45</td>
<td>965.46</td>
<td>F\textsuperscript{\textasciitilde}K\textsuperscript{\textasciitilde}F\textsuperscript{\textasciitilde}G\textsuperscript{\textasciitilde}K\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}G\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>17.4</td>
<td>20–29</td>
<td>1022.44</td>
<td>1022.47</td>
<td>F\textsuperscript{\textasciitilde}O\textsuperscript{\textasciitilde}F\textsuperscript{\textasciitilde}K\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}F\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>18.2</td>
<td>20–30</td>
<td>1093.50</td>
<td>1093.52</td>
<td>F\textsuperscript{\textasciitilde}F\textsuperscript{\textasciitilde}O\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}F\textsuperscript{\textasciitilde}F\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>19.0</td>
<td>4–16</td>
<td>1638.76</td>
<td>1638.76</td>
<td>E\textsuperscript{\textasciitilde}E\textsuperscript{\textasciitilde}Y\textsuperscript{\textasciitilde}K\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>19.8</td>
<td>1–16</td>
<td>1953.87</td>
<td>1953.83</td>
<td>K\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>20.4</td>
<td>10–17</td>
<td>1052.54</td>
<td>1052.54</td>
<td>G\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}V\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
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<tr>
<td>22.3</td>
<td>4–17</td>
<td>1751.85</td>
<td>1751.81</td>
<td>E\textsuperscript{\textasciitilde}E\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}E\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>23.1</td>
<td>1–17</td>
<td>2066.96</td>
<td>2066.92</td>
<td>L\textsuperscript{\textasciitilde}V\textsuperscript{\textasciitilde}L\textsuperscript{\textasciitilde}</td>
</tr>
<tr>
<td>37.4</td>
<td>1–40</td>
<td>4327.00</td>
<td>4327.15</td>
<td>(Aβ\textsubscript{1–40})</td>
</tr>
</tbody>
</table>

NEP mediated hydrolysis was carried out as described in Table 2. The reaction was stopped by adding 10 μL of 5% TFA when approximately half of the substrate had been hydrolyzed (360 min.). The acidified reaction mixture was subjected to HPLC analysis as described in Table 4. Each peak was isolated and subjected to mass spectral analysis.

doi:10.1371/journal.pone.0032343.t006

Materials and Methods

Mutagenesis and production of expression vectors

NEP variants were constructed as gene segment cassette modules using degenerative oligonucleotide primers to introduce sequence diversity by PCR. Individual mutation cassettes were inserted into the pCDNA-shNEP-CHis (SacII+PstI) expression vector, a re-engineered pCDNA-3.1 vector with a silent SacII mutation introduced 5’ to the active site region within a secreted form of the human NEP (shNEP) coding sequence and a C-terminal hexahistidine affinity tag. Two silent mutations were made, using the Quickchange® II site directed mutagenesis kit (Stratagene) to eliminate additional PstI sites and facilitate cassette subcloning of the shNEP gene. A 3 kb fragment of lambda “stuffer” DNA was inserted between the SacII and PstI sites to allow gene segment cassette subcloning while eliminating wild-type sequences from being selected [27,28]. Non-polar substitutions at Phe\textsuperscript{563} were initially cloned into and sequenced using the pBPG1 vector, a re-engineered pCDNA-3.1 vector with a silent SacII “stuffer” DNA was inserted between the SacII and PstI sites to allow gene segment cassette subcloning while eliminating wild-type sequences from being selected [27,28]. Non-polar substitutions at Phe\textsuperscript{563} were initially cloned into and sequenced using the pBPG1 vector and subcloning into the pDNA-shNEP-CHis-(SacII-3kbstuffer-PstI) vector. PCR based mutagenesis was used to create NEP variants using degenerative primers containing appropriate restriction sites. For making polar substitutions at Phe\textsuperscript{563} the 5’ primer contained a PstI restriction site and the 3’ primer contained an NcoI restriction site to facilitate movement from the pBPG1 vector to the pDNA-shNEP-CHis-(SacII-3kbstuffer-PstI) vector. The degenerative primers (IDT DNA, Coralville, IA) used were:

- 5’-gatcgggtcggatcagatggg-3’ (S546 reverse primer)
- 5’-gggctcggatcagatggggttggagacagttgctgctatgtgctagcgg-3’ (S546 forward primer)
- 5’-ccctctggacaaccccVIfsptggtgcacggacagctcaac-3’ (F563 forward primer for non polar residues)
- 5’-ccctctggacaaccccVIdssptggtgcacggacagctcaac-3’ (F563 forward primer for polar residues)
- 5’-ccctctggacaaccccVIdaaggtgcacggacagctcaac-3’ (F563 reverse primer)
where V = A, C, or G; S = C or G; D = A, G, or T; B = C, G, or T; 
Y = C or T.

Expression and purification

NEP protein was expressed in HEK293T cells transfected with 
the pcDNA vector described above. Cells were grown in 
Dulbecco’s Modified Eagle Medium (DMEM, Gibco) containing 
10% FBS and 44 mM NaHCO3 added as a supplement. For 
transfections, Polyfector (BamaGen Bioscience) and plasmid DNA 
were incubated at room temperature for 20 min in serum free 
DMEM media and then added to HEK293T cells in the DMEM 
media. The media was replaced with serum free DMEM 12– 
14 hours post transfection, and collected 72–96 hours post 
transfection. To the media was added 1 M Tris-HCl, pH 7.4 to 
a final concentration of 50 mM and the secreted enzyme was then 
purified on a His-Select Affinity Agarose Column (Sigma). The 
affinity purification step yielded enzyme with the purity dependent 
on the level of NEP expression. Activities measured in this study 
were attributed to NEP, since a mock transfection and purification 
resulted in no activity toward any of the substrates tested and NEP 
inhibitors eliminated all activity. We estimated the amount of NEP 
protein present by running the purified preparations on 8% or 
10% SDS-PAGE gels along with purified NEP as a standard. The 
gels were stained with Sypro Ruby dye scanned on a Typhoon 
9400 Imager, and quantified with Image Quant 5.2 software. In 
preliminary experiments the gel was transferred to a polyvinylidene 
diure Fluoride (PVDF) and subject to Western blot analysis using 
goat anti-mNEP at 1:1000 (R&D systems) as the primary antibody 
and anti-goat IRDye800 at 1:20,000 (Rockland) as the secondary 
antibody. Probed membranes were imaged using an Odyssey 
irradier imager and Odyssey 2.1 software. Intensities of each band 
were analyzed with Image Quant 5.2 software. Data was analyzed 
using Prism4 software.

Activity assays

NEP activity was routinely assayed using the fluorogenic peptide 
glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (Glut-Ala-Ala- 
Phe-MNA, Sigma) [30]. Reactions of 400 µl contained 100 mM Glut-Ala-Ala-Phe-MNA, 1 µg of aminopeptidase [31] and 15 to 
100 ng of NEP or mutant NEP depending on their activity in 
20 mM MES buffer, pH 6.5. Activity was monitored with a 
Spectra Max Gemini XS plate reader using an excitation 
wave length of 340 nm and an emission wavelength of 425 nm. 
Reaction specificity was determined using the NEP inhibitors

Figure 4. Comparison of Aß1–40 cleavage between NEP, NEP563L, and NEP546E. A. HPLC cleavage profile of Aß1–40 cleavage by NEP and 
NEP mutants at ~30% hydrolysis. B. Rates of peak accumulation at each cleavage site normalized to that of NEP for mutant NEP546E. C. Rates of peak 
accumulation at each cleavage site normalized to that of NEP for mutant NEP563L. Dotted lines indicate the overall rate of hydrolysis of Aß1–40 from 
Table 2. Reactions were carried out at 37°C with 15 µM Aß1–40 in 20 mM MES, pH 6.5. 
doi:10.1371/journal.pone.0032343.g004
**Table 7. Accumulation rates of products of NEP dependent cleavage of Aβ1-40.**

<table>
<thead>
<tr>
<th>Single Cleavage</th>
<th>Peak</th>
<th>NEP</th>
<th>SS46E</th>
<th>F563L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleavage Site</td>
<td>Δarea/min/μg</td>
<td>Δarea/min/μg</td>
<td>Δarea/min/μg</td>
<td></td>
</tr>
<tr>
<td>G9-Y10</td>
<td>1-9</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>K16-L17</td>
<td>1-16</td>
<td>22</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>L17-V18</td>
<td>1-17</td>
<td>75</td>
<td>51</td>
<td>22</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Dual Cleavages</th>
<th>Peak</th>
<th>NEP</th>
<th>SS46E</th>
<th>F563L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleavage Sites</td>
<td>Δarea/min/μg</td>
<td>Δarea/min/μg</td>
<td>Δarea/min/μg</td>
<td></td>
</tr>
<tr>
<td>A3-F4,G9-Y10</td>
<td>4-9</td>
<td>10</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>A3-F4,K16-L17</td>
<td>4-16</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>A3-F4,L17-V18</td>
<td>4-17</td>
<td>9</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>G9-Y10,K16-L17</td>
<td>10-16</td>
<td>4</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>G9-Y10,L17-V18</td>
<td>10-17</td>
<td>18</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>E11-V12,L17-V18</td>
<td>12-17</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>F19-F20,K28-G29</td>
<td>20-28</td>
<td>11</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>F19-F20,G29-A30</td>
<td>20-29</td>
<td>16</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>F19-F20,A30-A31</td>
<td>20-30</td>
<td>34</td>
<td>15</td>
<td>26</td>
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</tbody>
</table>

Time course assays were carried out by incubation of NEP with 24 μM Aβ1-40 using reaction conditions as described in Table 5. At 0, 60, 150, 240, and 360 min, aliquots of 100 μL were removed followed by the addition of 10 μL of 5% TFA to stop further hydrolysis. Each reaction mixture was subjected to HPLC analysis as in Table 5 and peak areas measured. The rate of accumulation for each peak was calculated from the linear phase of the reaction.

doi:10.1371/journal.pone.0032343.t007

phosphoramidon and CGS 24592 [24], the latter being a highly specific and potent inhibitor.

**Kinetic Analysis**

Kinetic constants for NEP and its mutants were obtained using the assay conditions noted above, but with Glut-Ala-Ala-Phc-MNA varied from 20 to 500 μM. Typically 12 data points were obtained. The data were fit to the Michaelis-Menten equation using Prism4 software.

**Statistical Analysis**

Statistical analysis comparing wild-type NEP and its mutants was performed with Prism 4 software using a two-tailed paired t-test with a 95% confidence interval.

**Supporting Information**

**Figure S1** Sites Mutated in NEP. The active site region of the NEP-phosphoramidon complex [23] is shown with the protein in a ribbon and surface representation and the bound ligand in a stick representation. The mutant residue positions are in red with side chains shown. The zinc ion cofactor is represented by a yellow sphere. Phosphoramidon residues equivalent to substrate peptide positions P1–P2 are indicated. The approximate position of substrate P2 and P3 residues is shown by the blue ovals. Purple arcs indicate contact between the P1’ residue and F563.

**Figure S2** NEP and mutant NEPs produce similar levels of mRNA. Varying PCR cycles were used to estimate the relative amount of NEP mRNA of high and low expressing mutants. Total RNA was harvested from HEK293T cells 96 hrs post transfection and an equal amount of RNA was used as a template for first-strand synthesis to produce a cDNA library using an oligo(dT) universal primer. The cDNA libraries were then used as templates for PCR using primers specific for NEP (experimental) and β-Actin (control). Samples from PCR cycles 20, 25, and 30 were used to estimate NEP transcript levels. The NEP563K mutant product band intensity relative to NEP was 0.9, 1.0, and 1.3 at cycles 20, 25, and 30 respectively. NEP563V and NEP563L were at a level approximately half of the wild-type NEP transcript. In contrast, NEP563L exhibited the same activity as wild-type enzyme while NEP563V displayed ~25% of the wild-type activity, while the activity for NEP563K was undetectable (<1% relative to wild-type enzyme) under our assay conditions (Table 1).

**Figure S3** Determination of the concentration of NEP mutants. Purified NEP samples were subjected to SDS-PAGE on 8% polyacrylamide gels and stained for protein with Sypro Ruby dye (A). The gel contained 100, 250, and 500 ng of purified NEP, which was used to construct a standard curve (C) from which the concentration of each NEP form was calculated. Samples of purified NEP, NEP563V, and NEP563L were run at 15 μl and 30 μl. Intensities of each NEP band were fit to the standard curve (C) to give 8.9±0, 12.7±3.7, and 11.6±1.1 μg/μL for NEP, NEP563V, and NEP563L, respectively (E, solid bars). Similarly a

Proteomics Analyzer at the University of Kentucky Proteomics core. This facility is supported in part by grant P20RR020171 from the NIH/NCCR.

**Synthesis and analysis of NEP cDNA**

In order to compare NEP transcript levels, RNA from 96-hour post-transfected HEK cells was collected using a QiShredder column (Qiagen) and an RNasey Mini Kit (Qiagen). Using 5 μg of the harvested RNA, cDNAs were produced with a Superscript First Strand Synthesis kit (Invitrogen) using the oligo(dT) primer included in the kit. Using NEP specific primers (5’-aagtaagact-gaaga-3’ and 5’-tcttgatggtgctgac-3’) and primers for β-actin (5’-tagagccagagcagtaatc-3’) and 5’-tcttgagctaaccaac-3’) for controls, relative levels of cDNA were measured by comparing product formation at 20, 25, 30, and 55 cycles in PCR reaction comparing under standard conditions using 50°C annealing temperature.
Western blot derived from a 10% SDS-PAGE was run containing 10, 50, and 100 ng of purified NEP from which a standard curve was derived. D, NEP, NEPf563l, and NEPs546E were run at 3.75 μl and 7.50 μl. Intensities of each NEP band were fit to the standard curve (D) to give 11.0 ± 1.4, 13.3 ± 0.6, and 13.1 ± 2.2 ng/μl for NEP, NEPf563l, and NEPs546E, respectively (E, empty bars). Note - the difference in size between the NEP standard and the NEP experimental samples is due to differences in glycosylation between NEP isolated from CHO cells and HEK cells, respectively.

References

Acknowledgments
We thank the University of Kentucky Proteomics core, and particularly Dr. Carol Beach, for the mass spectrometry analysis presented in this study.

Author Contributions
Conceived and designed the experiments: TS DWR LHB LBH. Performed the experiments: TS LH. Analyzed the data: TS DWR LHB LBH. Contributed reagents/materials/analysis tools: TS DWR LHB LBH. Wrote the paper: TS DWR LHB LBH.