Investigating the turnover of N-acyl amino acids

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2 Abstract:

The metabolism of N-acyl amino acids (NAAs) is not fully understood, especially the enzymes responsible for their metabolism. The hypothesis examined in this thesis is that N-arachidonoyl glycine (NAGly) is metabolized by serine hydrolases, since it has a similar structure to anandamide, which is hydrolysed by the serine hydrolase fatty acid amide hydrolase (FAAH).This study aimed to investigate NAA metabolism using both a fluorometric assay and activity-based protein profiling (ABPP).
A fluorometric assay was developed to detect amino acids, mainly glycine, released from NAA hydrolysis using derivatization reagents such as (Naphthalene-2,3-carboxaldehyde NDC) and fluoresceine. Several conditions were investigated, for example, reagent concentration, pH, and solvent. While fluoresceine improved sensitivity, the overall assay showed low levels of fluorescent product and high variability, limiting its effectiveness for detecting NAA hydrolysis.

To address these problems, ABPP was used to identify active serine hydrolases in rat and human brain tissues. In human brain, several enzyme activities were detected, with some bands consistent with (Monoacylglycerol lipase)MAGL and FAAH based on molecular weight and inhibitor selectivity.

In rat brain tissue, however, none of the enzyme activities were significantly inhibited using a wide range of serine hydrolase inhibitors. NAGly failed to alter the activity of any enzymes detected with this method.

Overall, while ABPP is useful to detect multiple enzyme activities in complex proteomes, further refinements are needed for confident enzyme identification and determination of pathways of NAA turnover.

Abbreviations

ABBREVIATION	FULL NAME
ABHD6	α/β-hydrolase domain-containing 6
ABPP	Activity-based protein profiling
AEA	Anandamide
FAAH	Fatty acid amide hydrolase
LLOD	Lower limit of detection
MAGL	Monoacylglycerol lipase
NAAS	N-acyl amino acids
NAGLY	N-Arachidonoyl glycine
NARASER	N-Arachidonoyl Serine
NDC	Naphthalene-2,3-carboxaldehyde
RF VALUE	Retardation factor value
RFU	Relative fluorescence units

3 Introduction:

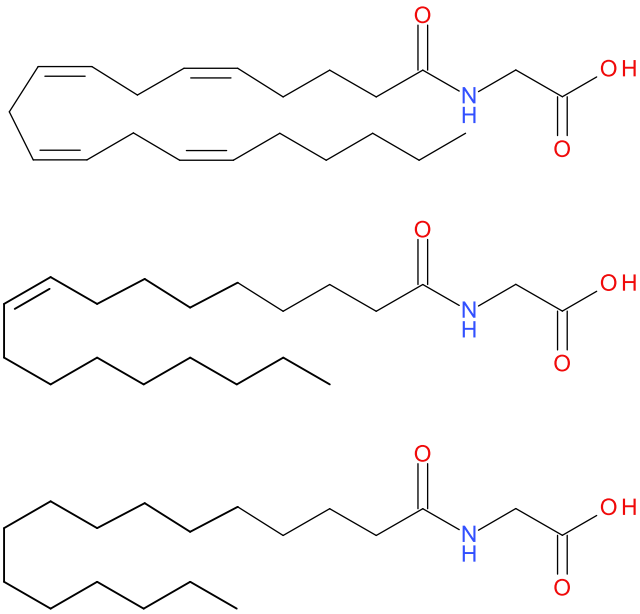
3.1 N-Acylamino acids history:

N-acylamino acids (NAAs) are a combination of protein and lipid metabolites in which an amide bond covalently links an amino acid to the acyl moiety of a long-, medium-, or short-fatty acid. NAAs belong to the family of endocannabinoid-like molecules but do not bind to cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) receptors. The first discovered example of the NAA group of compounds was N-arachidonoyl-glycine (NAGly)(see figure1). It was synthesised in an investigation of the structure–activity relationship of anandamide (AEA) – the first endocannabinoid discovered.(1)(2)

Using high-performance liquid chromatography/mass spectrometry (HPLC/MS/MS) in a targeted lipidomic method, 50 different types of NAAs from rat brain or bovine spinal cord samples were identified.(3) Examples of endogenous NAAs in the literature include N-arachidonoyl serine, N-arachidonoyl alanine, N-arachidonoyl taurine, N-oleoyl glycine, N-oleoyl serine, N-palmitoyl glycine, and N-palmitoyl serine.(4)

Consistent with a specialised role for them, N-acyl glycines were observed to vary between different tissues. N-arachidonoyl-glycine was found at higher levels in the spinal cord and small intestine than in the brain, kidney, and skin, while N-oleoyl glycine and N-docosahexaenoyl glycine were most abundant in the skin and/or lungs.(5)

Figure 1 The structures of N-acylglycines. In descending sequence, N-arachidonoyl glycine (NAGly), N-oleoyl glycine, and N-palmitoyl glycine.



3.2 Potential association with human diseases/disorders

Two broad metabolomic-wide association studies showed that high levels of N-oleoyl serine and N-oleoyl leucine in human plasma were linked with the future risk of coronary heart disease and heart failure, respectively.(6)Plasma levels of N-oleoyl-glycine, N-oleoyl-serine, N-oleoyl-leucine and N-oleoyl phenylalanine of 2,351 black adult participants of the Jackson Heart Study –a prospective cohort study that quantified the plasma levels of NAAs between 2000 and 2004 and two follow-up visits (2005–2008 and 2009–2012) – identified that N-oleoyl-glycine and -serine were associated with protective traits, showing inverse associations with cardiometabolic diseases like diabetes, while N-oleoyl leucine and N-oleoyl phenylalanine correlated with cardio metabolically disadvantageous traits, such as high body mass index (BMI) and waist circumference.(6)(7)Also, a study showed elevated NAGly in parathyroid tumours compared to adjacent tissues, suggesting that NAGly could be a parathyroid tumour biomarker. In addition, another study showed feeding-dependent changes in plasma NAGly, which had an anti-inflammatory effect on T helper cells by reducing Th1 and Th17 cytokine levels in CD4+ T cells isolated from obese subjects.(8)

3.3 Biological activity of NAAs

3.3.1 GPR18 as a possible NAGly receptor

Before describing the biological activity of the NAAs, it is important to discuss the receptors through which the NAAs are proposed to act. The SCREEN-WELL® Bioactive Lipid Library contains bioactive 198 lipids. Examples of these bioactive lipids are NAGly, AEA, phytocannabinoids, and fungal metabolites.(9) These lipids were screened by measuring intracellular Ca^{2+} mobilisation in GPR18-expressing polyclonal L929 cells (derived from the cell lining of a mouse's connective tissue) because they are suitable

for transfection.(10) NAGly at 10μM stimulated a significant increase in the intracellular concentration of Ca2+ in these cells compared with that in mock-transfected L929 cells.(11)Therefore, NAGly was proposed to be an endogenous ligand of GPR18.

A study in 2010 demonstrated that (NAGly) stimulated the directed cell migration via the activation of receptor GPR18 in BV-2 microglia. Migration was observed in GPR18-expressing cells treated with NAGly, while it was not seen in control cells lacking GPR18.(12)Two years later, the same authors reported that siRNA decreased GPR18 expression in BV-2 microglia (knockdown), and the result was decreased migration response to NAGly, which added further weight to the previous hypothesis.(13)

NAGly was reported to have an anti-inflammatory effect by stimulating prostaglandin E2 and leukotriene B4 levels through cyclooxygenase and lipoxygenase enzymes, contributing to the resolution phase of the inflammation.(14) In addition, NAGly decreased the migration of inflammatory cells into a site of acute inflammation.(14)GPR18 is highly expressed in T and B cells and was suggested to play a role in the anti-inflammatory effect of NAGly.[9]The chronic autoimmune disease rheumatoid arthritis (RA), is characterised by synovial inflammation and joint degeneration.(15)

A subsequent study reported that NAGly was not a ligand for GPR18 in native neuronal systems, with no evidence for Gαi/o-protein signalling.(16) An independent study using a high-throughput screen of 43 lipid ligands, such as AEA and NAGly, based on the ß-arrestin PathHunter™ cell-based assay of recombinant G protein-coupled receptors, showed no activation of the GPR18 receptor.(17) Ghayth Abdulrazzaq, during his PhD studies in this laboratory (2017) was unable to find NAGly (or THC) activation of recombinant GPR18. Therefore, there is a high probability that NAGly is not a ligand of the GPR18 receptor.

3.3.2 Other potential molecular targets of NAAs

In a study on the human glycine transporter 1b (GLYT1b) and human glycine transporter 2A (GLYT2a), NAGly and N-arachidonoyl-L-alanine (NAraAla) reversibly inhibited the glycine transporter GlyT2/SLC6A5, decreasing glycine uptake by presynaptic glycinergic neurons of the spinal cord and increasing glycinergic neurotransmission, which resulted in an analgesic effect.(18) The proposed mechanism of analgesia is that the normal sensation via activation of these glycinergic receptors in the dorsal horn of the spinal cord produces a strong, feed-forward inhibition of protein kinase Cγ, which prevents non-noxious sensory information from reaching the neurokinin 1 receptor, which sends this information to the brain.(19)

N-arachidonoyl-L-serine regulated cytoskeleton (actin) of the cerebral microvascular endothelium derived from human brain through activation of CB1, CB2, and transient receptor potential cation channel subfamily V member 1(TRPV1) receptors leading to reduced Rho/ROCK associated kinase and stimulated Akt kinase by

phosphorylation (20). The activation of RhoA stimulates the transcription of angiogenic genes, such as VEGF and FGF, as well as angiopoietins. These genes encode proteins responsible for the formation of new blood vessels.(21) This controls the organization of the cytoskeleton, which leads to an increase in angiogenesis.C2C12 myoblasts are cells that have been used mainly to study the process of skeletal muscle differentiation.(22) In a study on C2C12 cells with oligomycin, an ATP synthase inhibitor, and N-oleoyl-L-phenylalanine (NOlPhe), oligomycin alone increased the mitochondrial membrane potential, while NOlPhe decreased the mitochondrial membrane potential. Therefore, NAA stimulates uncoupled respiration in the mitochondria and disturbs the electron transfer chain in adipose tissue, similar to the uncoupling protein 1(UCP1) but independently. The result of the mitochondrial uncoupling is heat production instead of ATP production, which means that NAAs have thermogenic effects.(23)

In a study on mouse pancreatic islets, monitoring intracellular Ca2+ levels, NAGly closed KATP channels on the cell surface of beta cells, potentiating membrane depolarisation and the opening of voltage-gated Ca2+ channels, leading to exocytosis of insulin-containing vesicles. (24)As such, NAGly may be influential through an unknown molecular target for glucose homeostasis.

NAGly is reported to act as a vasorelaxant in rat-isolated small mesenteric arteries by activating an unidentified Gi/o-coupled receptor, which leads to the activation of phosphatidylinositol 3-kinase and Akt kinase, resulting in phosphorylation and activation of endothelial nitric oxide synthase.(25) A previous study showed that N-arachidonoyl serine activates BKCa channels directly in cultured cells26. This results in significant K+ efflux and cell membrane hyperpolarisation, followed by vasorelaxation. (25)

An uncorroborated study showed that N-palmitoyl glycine, N-oleoyl glycine and N-linoleoylglycine stimulated signaling in cells expressing the orphan receptor GPR132. Activation was measured through intracellular signaling assays (e.g., calcium, reporter systems). (26)

3.4 Regulation of NAA levels:

3.4.1 Synthetic routes for NAAs

The enzymes that are responsible for the synthesis of NAAs are not fully understood yet, except for proposed routes for production of NAGly.(2) Oxidation of the endocannabinoid AEA sequentially through alcohol dehydrogenase and aldehyde dehydrogenase leads to the production of NAGly. Another potential pathway is that arachidonoyl-coenzyme A (CoA) and glycine undergo a condensation reaction in the presence of cytochrome-c. (2)(27) A similar pathway has been described for N-arachidonoyl γ-aminobutyric acid (GABA), serine and alanine, and N-oleoyl glycine, serine, alanine and GABA.[4] It has been proposed that liver mitochondria accumulate fatty acyl-CoA, which undergo this oxidative process.(28)

Another enzyme, glycine N-acyltransferase, first discovered in mammalian liver and kidney mitochondrial fractions, conjugates benzoic acid and short- and medium-chain fatty acids to glycine. (29) Recombinantly expressed human glycine N-acyltransferase-like 2, which is considered a detoxifying enzyme that conjugates glycine to a variety of substrates, was described to prefer the substrate oleoyl-CoA (C18:1) more than arachidonoyl-CoA. Therefore, this suggests it might be responsible for the synthesis of N-oleoyl glycine. (29)

PM20D1A is a newly discovered, poorly characterised enzyme.(30) Gene expressed is evident in multiple tissues in mice, such as the liver, bladder, brain, large intestine, pancreas, kidney and heart, while in humans, its highest expression is in the pancreas and skin, but it is also expressed in several other tissues. (31) This enzyme is closely associated with low- and high-density lipoproteins, as it travels through the bloodstream. These lipoproteins serve as both NAA biosynthesis sites and coactivators of PM20D1 activity.(32) PM20D1 is an enzyme with two opposite actions: catalysing fatty acid and amino acid conjugation reactions to produce NAAs and the reverse hydrolytic reactions.(31)(see figure 2)

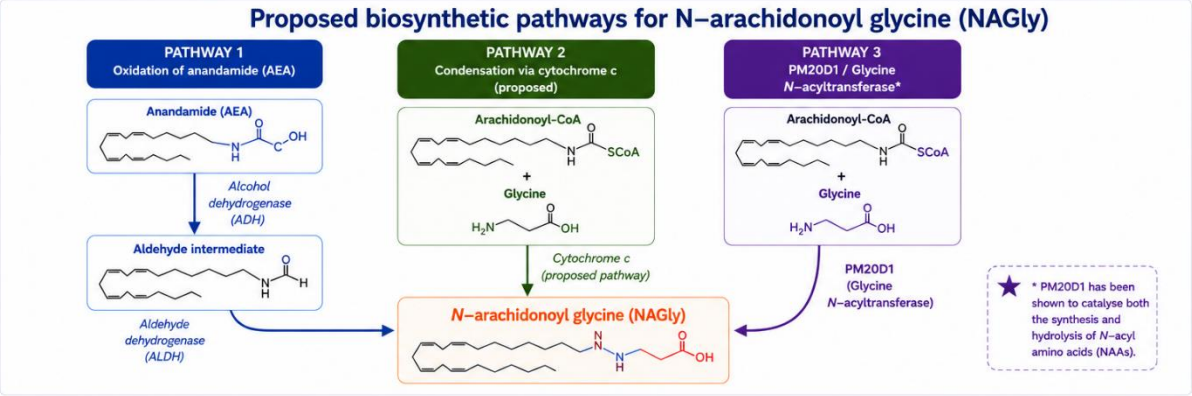


Figure 2 :proposed routes of synthesis of NA-Gly

3.4.2 Hydrolysis of NAAs

Several enzymes are reported to contribute to the hydrolysis of NAAs. One example is FAAH, a microsomal enzyme responsible for the hydrolysis of AEA and other fatty acid ethanolamines.(33) FAAH hydrolyses NAAs (N-AraGlycine , N-AraSerine, N-AraAlanine) to their corresponding amino and arachidonic acids. Therefore, NAAs are substrates of the FAAH. Conversely, NAAs have been considered as inhibitors of FAAH, since they inhibit the binding of AEA to the FAAH.(2) A study on FAAH activities in human, mouse and rat brains examined the inhibitory effects of NAGly, Ala, Val, Leu, Ile, Glu, Gln and Asp on FAAH.(33) Only NAGly, Ile and Ala inhibited FAAH activity. However, this inhibition was species dependent. NAGly inhibition of human FAAH was weaker, unlike the mice

and rat FAAH, while NAraAla inhibition of human FAAH was higher than mice FAAH.(33) NPAlGly was reported to be up-regulated in FAAH-ko mice.(34)

N-Oleoyl Glycine (NOleGly) is hydrolysed by peptidyl-glycine-alpha-amidating monooxygenase (PAM), NOleGly undergoes oxidative cleavage to the primary fatty acid amide: oleamide, which may function as a local hormone/mediator. A defect in PAM enzyme leads to a high concentration of N-acyl glycine in the urine.(35)

N-arachidonoyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) is the enzyme primarily responsible for producing AEA.(4) NAPE-PLD knockout mice brains demonstrated decreased blood levels of both AEA and N-AraSerine; however, the level of NAGly increased in four regions of the brain, which suggests that NAPE-PLD may be a hydrolytic enzyme and not a synthetic enzyme of NAGly.(4) (See figure 3)

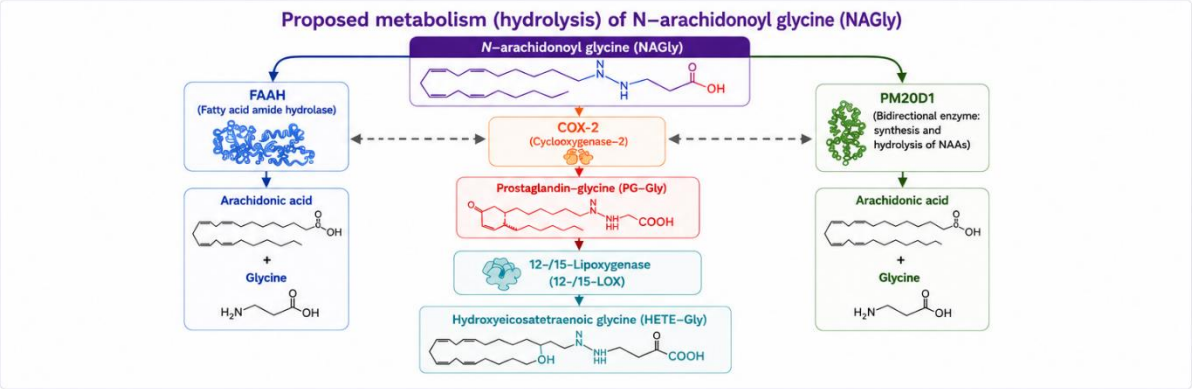


Figure 3:proposed routes of metabolism of NA-Gly .

3.4.3 Biotransformation of NAAs

In addition to the synthetic pathway and the hydrolysis of NAAs, it has been discovered that NAAs undergo biotransformation. Cyclooxygenase-1 (COX-1) and COX-2 metabolise the arachidonoyl group in AEA, 2-arachidonylglycerol and N-arachidonoyl -amino acids to prostaglandin analogues.(4)(36) COX-1 is expressed in almost all tissues, while COX-2 expression increases in response to triggers, such as cytokines and mitogens.(37) COX-2, specifically not COX-1, metabolises NAGly to prostaglandin-glycines (PG-Gly). Mutagenesis studies have shown that the Arg-120, Glu-524 and Arg-513 residues responsible for COX-2 selectivity and binding to oxidise NAGly, NAGly to PG-Gly. NAraAla and N-arachidonoyl GABA undergo low rates of oxidation by COX-2 but undergo high oxidation by COX-1.(38)

12- and 15- lipoxygenases oxidise polyunsaturated fatty acids, including the arachidonoyl group in NAGly, NAraAla, and NAraGABA, to 12- and 15-hydroxy eicosatetraenoic glycine, alanine, GABA.(4)

3.5 Serine hydrolase regulation of the endocannabinoid system:

The metabolic regulation of NAAs appears deeply connected with the "classical" endocannabinoid system. These enzymes belong to a big network of serine hydrolases regulating N-acylethanolamines (NAEs), such as AEA, and monoacylglycerols (MAGs), such as 2-AG.(39)The first step in the synthesis of the anandamide is *N*-arachidonoyl phosphatidylethanolamine (NAPE) by *N*-acyltransferase that followed by the cleavage of *N*-arachidonoyl-PE to produce anandamide, This step is catalyzed by NAPE-phospholipase D. (40)(41) Hydrolysis of AEA is mediated by fatty acid amide hydrolase (FAAH), that is a ~60 kDa integral membrane-associated enzyme mostly located in intracellular membranes.(42)(43) Selective inhibitors, such as URB597 and JNJ1661010, have been used to define the action of FAAH.

2-AG synthesis is through diacylglycerol lipases (DAGL). The hydrolysis of 2-AG is predominantly by MAGL which is a smaller enzyme (~33 kDa) that is primarily cytosolic, although it can also associate with cellular membranes, (44)It is responsible for the majority of 2-AG hydrolysis in the brain, producing arachidonic acid and glycerol and thereby regulating both endocannabinoid signaling and downstream lipid pathways. Selective inhibition of MAGL, for example, by JZL184, results in elevated 2-AG levels.(45)α/β-hydrolase domain-containing 6 (ABHD6) is a membrane-associated enzyme (~35 kDa) that contributes to 2-AG hydrolysis within specific cellular compartments. Although it accounts for a smaller proportion of total 2-AG breakdown than MAGL, it is thought to play an important role in fine-tuning endocannabinoid signaling at the local level. Pharmacological inhibition of ABHD6 can be achieved using compounds such as WWL70 and KT203.(46)

These enzymes share a conserved catalytic mechanism based on a Ser–His–Asp triad, in which the serine residue acts as a nucleophile to attack the substrate's carbonyl group, forming a transient intermediate that is subsequently hydrolyzed to release the products and regenerate the enzyme. Substrate binding is largely driven by hydrophobic interactions that position lipid substrates within the active site, while membrane association, particularly for FAAH and ABHD6, facilitates direct access to substrates embedded within lipid bilayers(see Figure4).(47)

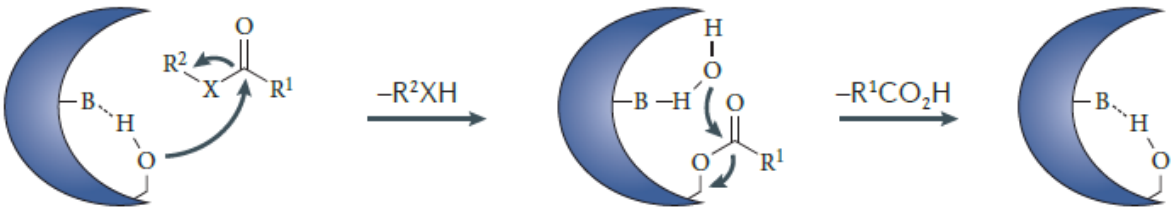


Figure 4 :The serine hydrolase catalytic cite

Activity-Based Protein Profiling (ABPP) uses covalent probes to monitor the functional state of multiple enzymes simultaneously. (48)Fluorophosphonate (FP) Probes: Efficiently label FAAH, MAGL, and ABHD6. (49)The lactone probes MB064 was synthesized specifically because DAGL is not efficiently labeled by FP probes. (50)

3.6 Hypothesis and aim:

- Anandamide, NAraGly and 2AG have common acyl sidechains, suggesting that they could be metabolized through common pathways
- This study aims to investigate the potential for FAAH (or other serine hydrolase activities) to metabolize NAGly.

3.7 Objectives:

1. To quantify NAGly hydrolysis through fluorometric detection of the amino acid product
2. To apply the ABPP technique to identify enzyme activities in rat and human brain tissue that are reduced in the presence of NAGly (and other N-acylamino acids)

4 Chapter 2: Methodologies

4.1 Fluorometric Assay

A previous PhD student in the lab examined N-acylethanolamine hydrolysis, particularly on AEA, using derivatisation and detection of the generated ethanolamine.(51) This project continues that investigation, focusing on adapting the methodology to measure amino acid production, particularly glycine detection, from NAA hydrolysis.

4.1.1 Derivatisation of glycine with NDC

The first derivatisation agent used was naphthalene-2,3-carboxaldehyde (NDC). NDC dilutions from 0.5-4 mg/ml were prepared in methanol or acetonitrile, then further diluted 1:4 in 0.1 M $KHPO_4$ buffer (pH = 11.5). Serial dilutions of glycine were initially prepared with deionized water and then diluted in $KHPO_4$buffer (pH = 7.4) to give seven concentrations (1000-0.1 µM). 50 µl of each dilution was distributed individually to 96 well plates, and then 150 µl of the NDC solution was added. After 30 minutes, the fluorescent product using a Molecular Devices Spectramax 2e (excitation 420 nm/emission 480 nm). Different sulfite concentrations were added to the NDC to promote fluorescent adduct formation.

4.1.2 Derivatisation of glycine, taurine, alanine and serine with fluorescamine:

Fluorescamine dilutions from 1.5-6 mg/ml were prepared in methanol. Serial dilutions of glycine, taurine, alanine and serine were prepared in deionized water (1000-0.1 mM) and then diluted with $KHPO_4$buffer (pH = 7.4) or $KHPO_4$ buffer (pH = 9.2). 100 µl of each dilution was distributed individually to 96 well plates, and then 2 µl of the fluorescamine solution was added. After 30 minutes, the fluorescent product was quantified using a Molecular Devices Spectramax 2e (excitation 390 nm/emission 475 nm).

4.2 Activity-based protein profiling

4.2.1 : Tissue preparation (conducted by SPH Alexander):

Rat (male Sprague-Dawley, 150-250 g) brains were obtained after Schedule 1 killing and stored at -80 °C. The whole brain was thawed and homogenized in 8 volumes of 10 mM Tris: 0.2 mM EDTA, pH 7.4 buffer using a glass: Teflon homogenizer. The suspension was then centrifuged at 20,000 g for 30 min at 4 °C. The supernatant layer was removed and stored. The pellet was homogenized again in the same volume of buffer using the glass: Teflon homogenizer, and the centrifugation step was repeated. The supernatant layer was removed and combined with the previous supernatant and then stored as aliquots at -80 °C. The pellet was resuspended in 6 volumes of the same buffer and then stored as aliquots at -80 °C.

4.2.2 Cell culture materials:

SHSY5Y cells: human neuroblastoma cell line, the range of passage number is between 3 and 15 passages.
PBS: divalent cation free phosphate-buffered saline.
DMEM /F12/12 50/50,1x: Dulbecco's Modified Eagle's Medium/Ham's F-12 50/50 Mix without L-glutamine, was purchased from Corning company (LOT 3182203).
FBS (Fetal Bovine Serum**):** Trypsin-EDTA solution was purchased from Sigma Aldrich (Lot:0000267209).

4.2.3 Cell Culture Method:

SHSY5Y cells were cultured in T175 cm^3 flasks. The cells were washed with PBS, then 5 ml Trypsin EDTA was added, followed by the addition of 5 ml DMEM /F12/12 50/50,1x media to which was added 50 ml of FBS,5 ml L-glutamine, 5 ml of sodium pyruvate solution, and 5 ml of non-essential amino acids to 500 mL of media. All the content was transferred from the flask into the 30 ml universal tube. The cells were centrifuged for 5 minutes at 1000 rpm. The supernatant layer was removed, and the pellet was resuspended with 6 ml of DMEM media. 2 ml of the cells were added to each of the three T175 flasks, and 40 ml of the DMEM media was added to each T175 flask.

4.2.4 Cell harvesting and fractionation

PBS was added to two-T-175 flasks at 90% confluency; the cells in the flask were scraped, and the cells were collected into a 50 ml universal tube, and centrifuged for 5 minutes at 1000 rpm. The supernatant layer was removed, and the pellet was resuspended with 500 µl 10 mM Tris, 0.2 mM EDTA pH 7.4. The cell suspension underwent homogenization by sonication (Misonix) on ice for 3 cycles of 10 s and 15 s rest. The cell suspension was centrifuged for 30 minutes at 20 000 g at 4 °C. The supernatant layer was collected and the pellet was resuspended with 500 µl of 10 mM Tris, 0.2 mM EDTA, pH 7.4. A second cycle of sonication and centrifugation provided soluble and particulate fractions. The latter was resuspended again with 500 µl 10 mM Tris 0.2 mM EDTA, pH 7.4 by sonication.

4.2.5 Protein Determination:

The protein concentration was measured using the Pierce Bicinchoninic acid (BCA) assay from Thermo Scientific Company (LOT VA295774). The working reagent was prepared by mixing 50 parts of Pierce BCA reagent A with 1 part of Pierce BCA reagent B (50:1, Reagent A, B). 200 µl of this mixture was added to wells in a 96 well microplate, then 25 µL of 1 to 5 diluted protein was added, the microplate was shaken for 15 minutes then incubated at 37 °C for 30 minutes. The protein concentration was detected by microplate reader from BMG Labtech company. Microplate reader settings:

- Wavelength: 562 nm
- Read mode: Absorbance (endpoint)

- Plate type: Clear flat-bottom
- Volume per well: 200 µL typical
- Incubation:
    - 30 min at 37 °C or
    - 2 hours at room temp
- Shaking: Optional before reading

4.2.6 Human post mortem brain samples

Human brains were obtained at autopsy in the Basque Institute of Legal Medicine, Bilbao, Spain (Erdozain et al., 2015). The study was developed in compliance with policies of research and ethical review boards at the moment of sample obtaining for post-mortem brain studies (Basque Institute of Legal Medicine, Bilbao, Spain). Samples from the prefrontal cortex (Brodmann's area 9) were dissected at autopsy and immediately stored at −70°C until assay. A toxicological screening on blood (psychotropic drugs and ethanol) was performed at the National Institute of Toxicology, Madrid, Spain. In all cases, a retrospective careful searching for antemortem medical diagnosis was performed, based upon DSM-IV or ICD-10. The mean demographic characteristics, PMD and RIN (RNA integrity number) of samples are detailed in Table 1.(52)

Table 1 Means of the age, post-mortem delay (PMD)of samples included in the study.

Age (years)	**50 ± 4**
PMD (hours)	21 ± 3

4.3 ABPP Materials:

Acrylamide/bis-acrylamide 30% solution was purchased from Sigma-Aldrich, APS (ammonium persulfate; Sigma-Aldrich, cat. no A3678), and TEMED (N,N,N',N' tetramethylenediamine; Sigma-Aldrich, cat. no T9281). The TAMRA-fluorophosphonate activity-based probe (FP-TAMRA) was acquired from Thermo Fisher, whilst Professor Mario van der Stelt of Leiden University in the Netherlands supplied MB064, synthesised by Dr. Marc Baggelaar.

4.4 ABPP Method:

ABPP is a powerful technique for discovering and screening small-molecule inhibitors against enzymes derived from cell lysates, subcellular fractions, tissues, and recombinant proteins. (53)An ABPP system uses a probe containing two parts: (1) a *reactive group* that binds and covalently alters the active sites of a large number of enzymes that share similar mechanistic and/or structural features, and 2) a *reporter tag*, such as a fluorophore or biotin, to enable detection, enrichment, and identification of probe-labeled enzymes through gel electrophoresis and in-gel fluorescence scanning

(gel-based ABPP), ABPP probes have been generated for over a dozen enzyme classes, including probes that react specifically with serine hydrolases(39), cysteine proteases(54), kinases(55), and glycosidases. (56) These probes make it easier to characterize changes in enzyme activity without changes in protein levels because they only recognize active enzymes and not their inactive versions.

The two probes used in this study are classified as fluorophosphonates (FP); an example of this group is TAMRA-FP, used to label and detect serine hydrolase activity; the second example is β-lactone-containing probes. An example of this group is MB064.(49)

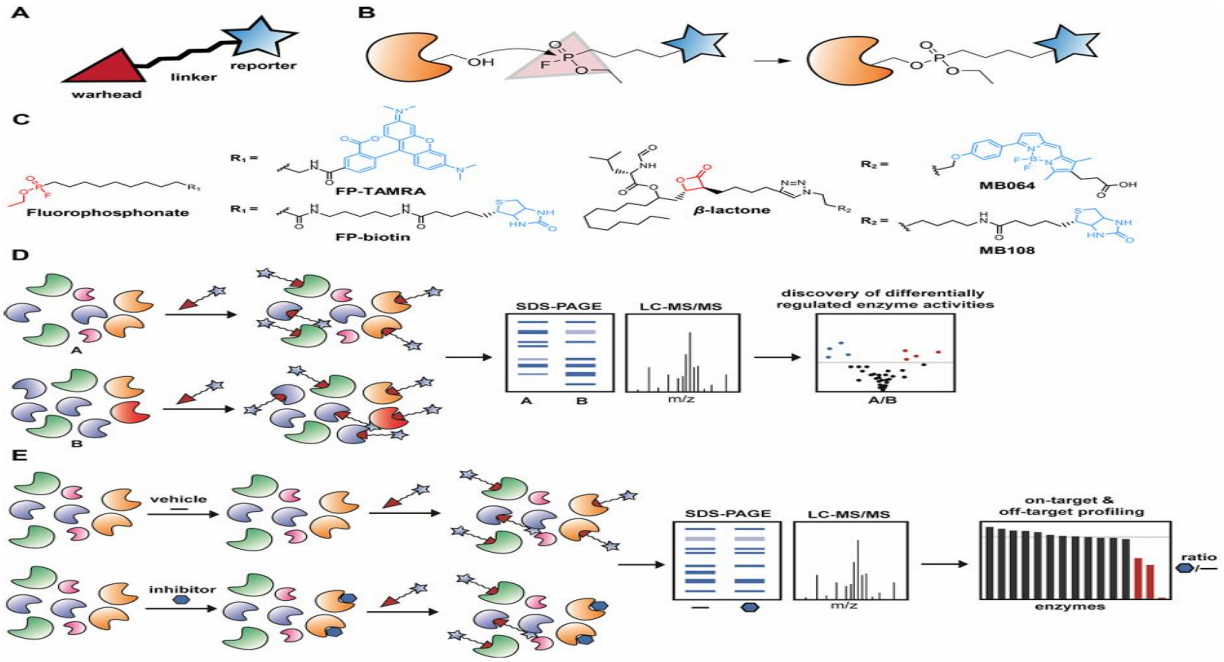
TAMRA-FP recognizes numerous serine hydrolase enzymes.(57) However, it cannot detect DAGL-α or DAGL-β. This lead to the synthesis of the MB064 probe37. While MB064 labels many fewer proteins than TAMRA-FP, it labels ABHD12 and ABHD6, alongside DAGL isoforms.

There are two types of ABPP (comparative and competitive activity-based proteome profiling). Comparative ABPP assesses enzyme activity of different proteomes; for example, samples of healthy and diseased cells or tissue. This could result in the discovery of possible novel therapeutic targets and help in the study of the effect of pharmacological intervention on the activity of the enzyme. In contrast, competitive ABPP samples are pretreated with inhibitors before adding the probe. A decrease in fluorescence intensity of the bands reflects that the inhibitor interacted and blocked the enzyme's active site. This approach is useful in determining inhibitor potency and selectivity.(44)

4.4.1 Sample reactions with the activity probes:

16 µL of the tissue fractions were incubated at 37 °C for 15 min to allow metabolism of any endogenous substrates; two µl of inhibitor/competitor (for example, NaGly) were added, and immediately, the probes were added: 2 µl of FP-TAMRA (500 nM) at 37 °C for 30 minutes or 2 µl of MB064 (250 nM) at 37 °C for 60 min. After the

incubation,4 µl of 6x Laemmli buffer was added to stop the reaction. The samples were stored at -20 °C before they underwent gel electrophoresis.(See Figure 5)



4.4.2 Gel electrophoresis:

Figure 5: The main concept of ABPP.

A)General structure of an activity-based probe consisting of a reactive warhead, a linker, and a reporter tag used for detection or enrichment.
(B) Mechanism of probe labelling, showing the covalent reaction between the fluorophosphonate probe and the active-site serine residue of a serine hydrolase.
(C)Examples of commonly used activity-based probes for serine hydrolases, including fluorophosphonate (FP)-based probes (FP-TAMRA and FP-biotin) and β-lactone-based probes (MB064 and MB108).
(D) Comparative ABPP workflow for identifying differences in enzyme activity between two biological samples. Following probe labelling, proteins are analysed by SDS-PAGE and/or LC–MS/MS to identify differentially regulated enzyme activities.
(E) Competitive ABPP workflow used to evaluate inhibitor selectivity. Samples are pre-incubated with either vehicle or inhibitor before probe labelling, allowing identification of on-target and off-target enzyme inhibition by SDS-PAGE and LC–MS/MS.

In this experiment, 20 cm × 20 cm gels (10% acrylamide, Table 1) were used. The stacking gel made up around one-third of the gel, with the remaining two-thirds being the resolving gel. Up until the dye front eluted at the bottom of the gel, a constant current was applied. Two molecular markers were used over the course of these experiments ranging from 17 to 250 kDa (Rainbow recombinant protein RPN800E, Lot:17976687 ECL plex fluorescent marker, RPN851E, Lot: 18112176).

Table 2 Gel composition for making a single 10 % acrylamide gel.

Addition	Resolving gel (10 %)	Stacking gel (4 %)
Resolving gel buffer (1.5 M, pH 8.8)	8.75 mL	
Stacking gel buffer (0.5 M, pH 6.8)		3.8 mL
Distilled water	14.35 mL	9 mL
SDS (10 %)	350 µL	150 µL
Acrylamide (30 %) Lot	11.55 mL	1.98 mL
TEMED (tetramethyl ethylenediamine)	17.5 µL	15 µL
APS (ammonium persulfate) 10% (w/v)	175 µL	75 µL
Total	**35 mL**	**15 mL**

4.4.3 Gel Organization and Sample Loading

Gel scanning and band quantification: Before the glass plates containing the gel were placed in the Amersham Typhoon scanner for imaging, their surfaces were cleaned and carefully dried with tissue paper. A dual scan using the Cy3 filter (532 nm excitation, 580 nm emission) and Cy5 channels (635 nm excitation, 665 nm long-pass emission filter) was selected for the scanner. A TIFF file was created from the scanned image. Following the scan, the gel was removed from the glass plates and proteins in the gel were stained with Coomassie blue.

This functions in three phases: fixation, staining, and destaining.

All three phases make use of a solvent mix, which can be stored for months at room temperature: 100 mL aceticacid,400 mL water, and 500 mL methanol.

The **staining solution** was made up by dissolving 500 mg Coomassie Blue R250 (**not** G250) in 500 mL of the solvent mix. This needed to be stirred for some time (at least an hour) and filtered before use. It can be used for an extended period. If a precipitate appears, a fresh staining solution should be prepared.

Gels are handled only with gloved hands for two reasons: it is possible that not all the acrylamide polymerises and may be transferred to the skin. Second, fingerprints on the gel scans are annoying and may interfere with band quantification.

4.4.4 Gel analysis protocol:

1) Use Image J to crop and adjust the brightness and contrast.
2) Open Image processing and lane/band detection using Image Lab Ensure the bands are within the lane by adjusting the top and the bottom. Add the bands for the molecular weight marker and check the lane profile. Add the bands for the second

lane and check the lane profile. Keep the same number of bands for lanes that are from the same sample and the same probe, then check with the lane profile.(see figure4)

3) In the Excel sheet, compare the RF, which is the ratio of the distance travelled by that band to the dye front, and the adjusted volume of the bands that belong to the lane of the same sample and probe, under the same condition.
4) The RF mean of the particulate fraction was summarized in one Excel sheet to compare the number of bands between each sample to make the next step easier. Also, there will be a comparison between the calculated number of bands by image lab software and the visual number of bands, which will help us in labeling the bands alphabetically (see Figure 4).

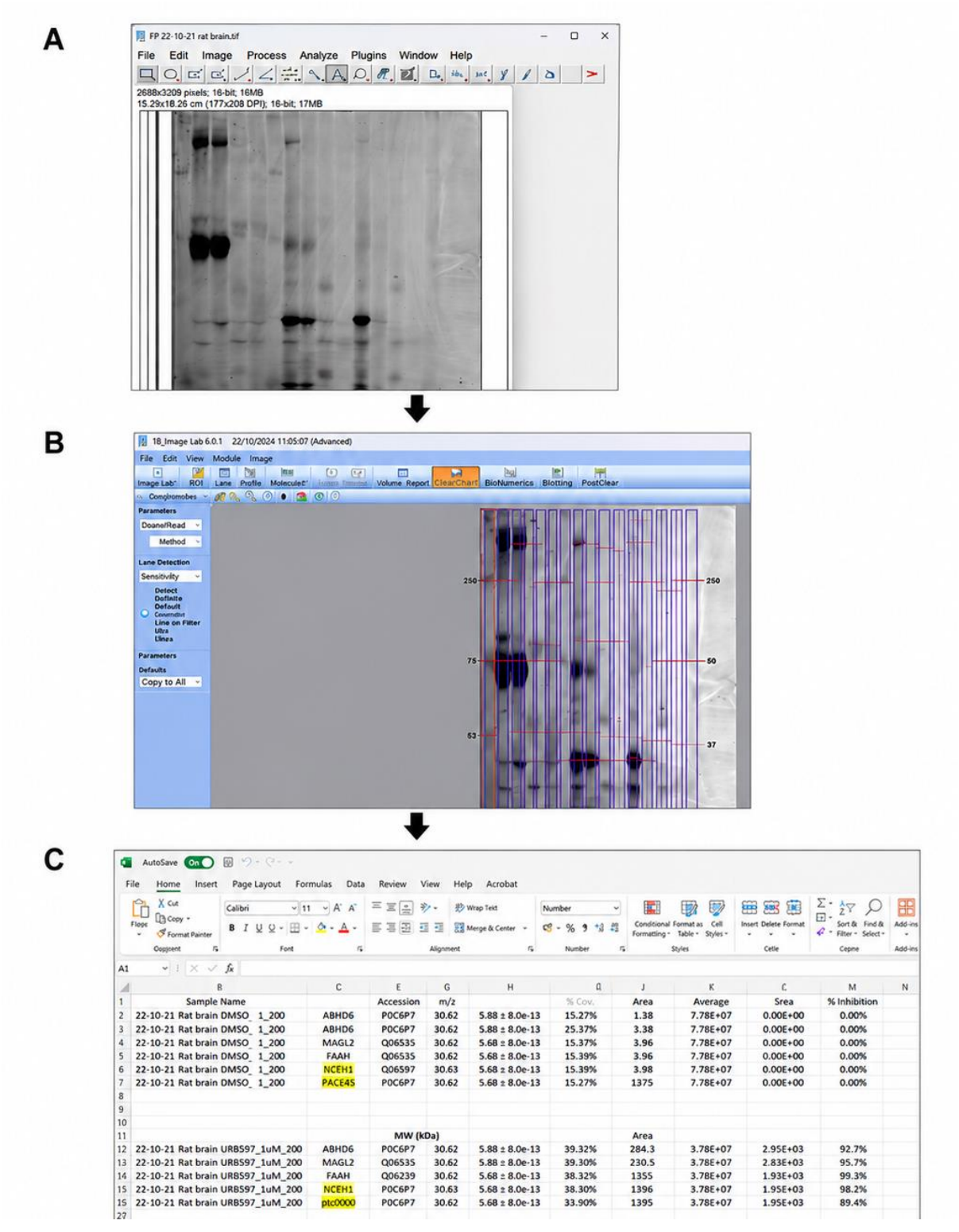


Figure 7 flow chart of gel analysis

A)Image J to crop and adjust the brightness and contrast of the Raw in-gel fluorescence image obtained following activity-based protein profiling (ABPP) of rat brain membrane proteomes labelled with the FP-biotin probe.B)Image processing and lane/band detection using Image Lab software. Molecular weight markers (kDa) are indicated to

facilitate band identification)Export of quantified band intensity data to Microsoft Excel for analysis.

5 Chapter 3: Detecting N-acyl amino acid hydrolysis by fluorometric enzyme assay

5.1 Introduction:

This section focuses on the optimization of a fluorometric test designed to detect N-acyl amino acid (NAA) breakdown by measuring the release of amino acids, particularly glycine.

Fluorometric assays are favoured because they give a sensitive and simple way to understand biochemical reactions. This advantage increases when combined with derivatization techniques that convert non-fluorescent compounds into fluorescent products. In this study, two derivatization methods were evaluated: naphthalene-2,3-carboxaldehyde (NDC) and fluorescamine. Both reagents react with the primary amine groups of amino acids to generate fluorescent compounds, enabling their detection and measurement with a fluorimeter.(58)

The main aim of this chapter is to evaluate the assay's performance in terms of sensitivity, selectivity, linearity, and reproducibility. The emphasis is on improving the limit of detection, increasing fluorescence intensity, and reducing background noise. To achieve this, multiple experimental conditions were investigated, including reagent concentration, buffer composition, pH, and the addition of 'developing substances' like sulfite.

5.2 Hypothesis:

There are currently a limited number of enzymes that hydrolyze NAAs, and most of the studies on human cells are limited. This project aims to identify the enzymes that hydrolyze NAAs and to identify their inhibitors in human cells. We hypothesize that other enzymes hydrolyze NAAs that have not yet been discovered in human cells.

The objective:

- To quantify the amino acid products of NAAA hydrolysis through a fluorometric enzyme assay using a derivatization agent.

5.3 Result:

What makes an assay useful is that it achieves accuracy, precision, selectivity, sensitivity, a low limit of detection (LLOD), and a low blank value. Accuracy means that the assay follows a gold-standard method, while precision means it gives the same result after each replicate, so the results are reproducible. Selectivity indicates the assay's ability to measure responses to the target substance only, while sensitivity reflects the small changes in test substance/target concentration that affect the assay result. The assay should not require a large number of additions to the samples to save the quantity of the chemicals, and the volume and size of the samples should be adjustable and easily changed. In addition, the assay's outcome should be attained in a measurable

range, in a short time and at a low cost. A useful assay that produces reliable results using a simple and intuitive procedure is desirable. In addition, it should be scalable and dependable, and have a wide dynamic range.

An assay's ideal outcome is to precisely determine the target substance's concentration in a sample with the lowest blank value and LLOD possible.

The assay is based on the quantification of the glycine produced from N-acyl glycine hydrolysis. The main principle of the assay is that NDC is electrophilic, so it needs a nucleophile to react with the amine group of the amino acid. The final product is a fluorescent adduct. (see Figure 7)

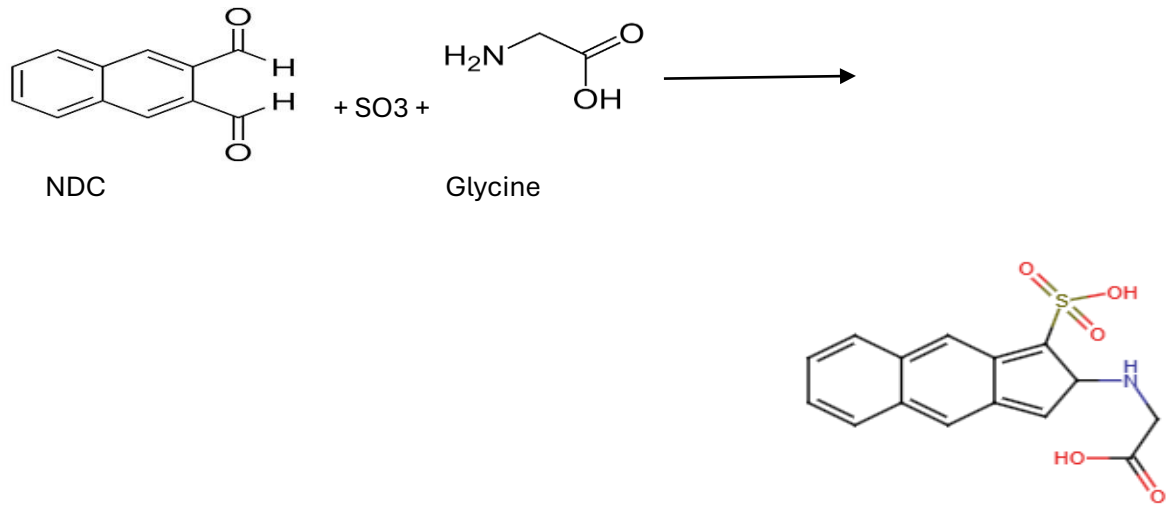


Figure 7 :The use of a nucleophile, which is sulfite, elevates the detection of glycine by increasing the fluorescence adduct.

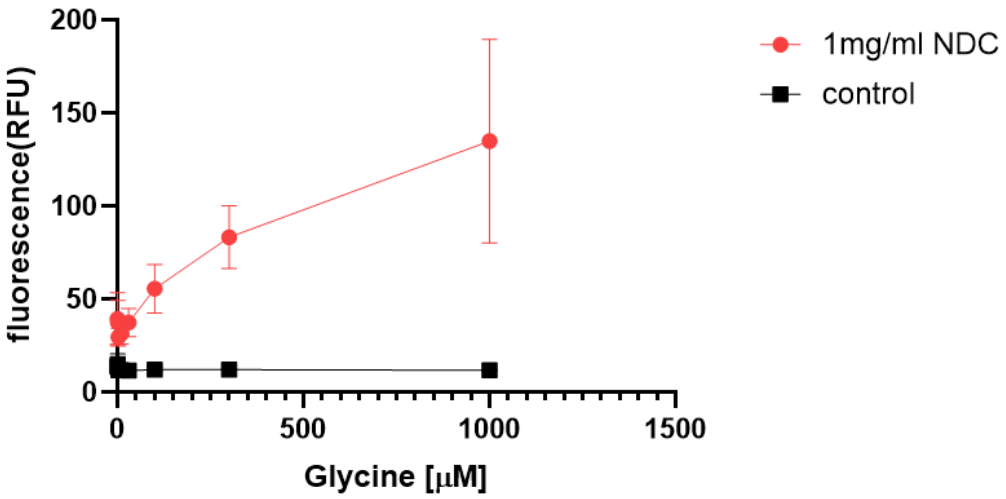


Figure 8 :Fluorescence development in the presence of a range of glycine concentrations in the absence and presence of 1 mg/mL NDC. I

Initially, the sensitivity to glycine was determined using a concentration response curve (figure 8).The results of the detection of glycine by NDC, the linear range of the fluorescence was between 39 to 134 relative fluorescence units (RFU), which is very low. The lower limit of sensitivity was 1 µM.

Therefore, the assay needs to be developed further to increase the fluorescence, since the assay would need to detect concentrations of glycine at submicromolar levels.

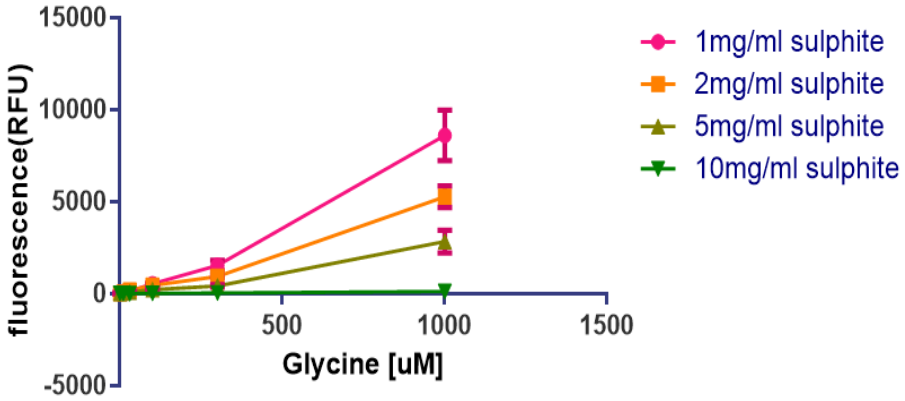


Figure 9 :Effects of sulfite on the development of fluorescence in the presence of glycine and NDC

To increase the fluorescence, we tested the effect of adding the four concentrations of sulfite (see Figure 9). 1 mg/ml sulfite showed the highest fluorescence. The linear range was between 350 and 8850 RFU. the LLOD was between 30 and 100µM of glycine, which indicated low sensitivity. The four concentrations of sulfite increased the fluorescence, but not to a sufficient fluorescence and with a higher blank value than without the additions of sulfite.

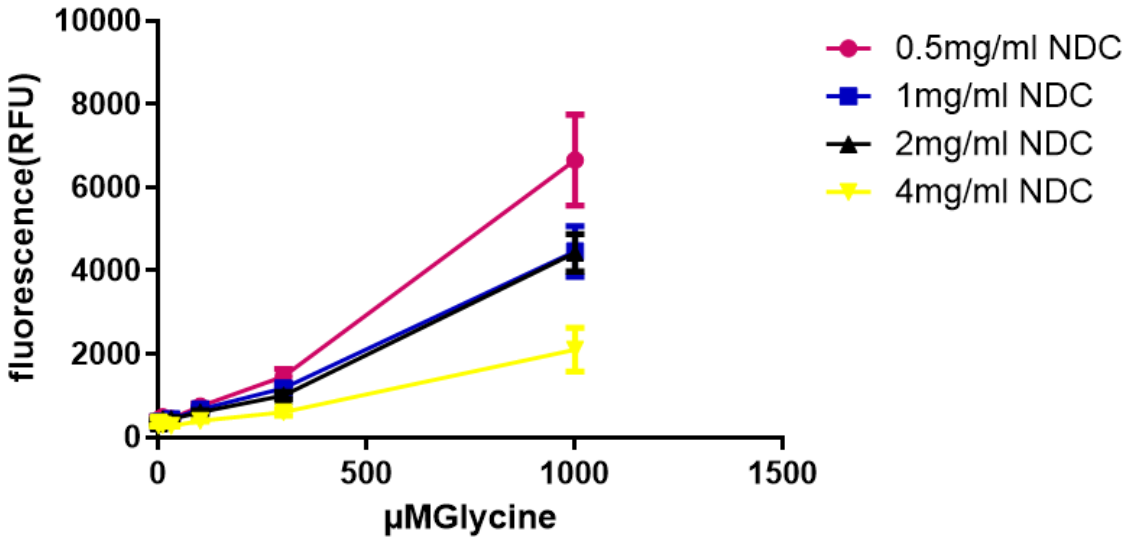


Figure 10 Effects of varying glycine concentrations on fluorescence production in the presence of different concentrations of NDC

To increase the fluorescence and sensitivity, we tested different NDC concentrations. 0.5 mg/ml of NDC showed the highest fluorescence (see Figure 10), but the LLOD was between 100 and 300 µM, which reflected low sensitivity. The fluorescence was still low, and the linear range was between 6665 and 12427RFU.

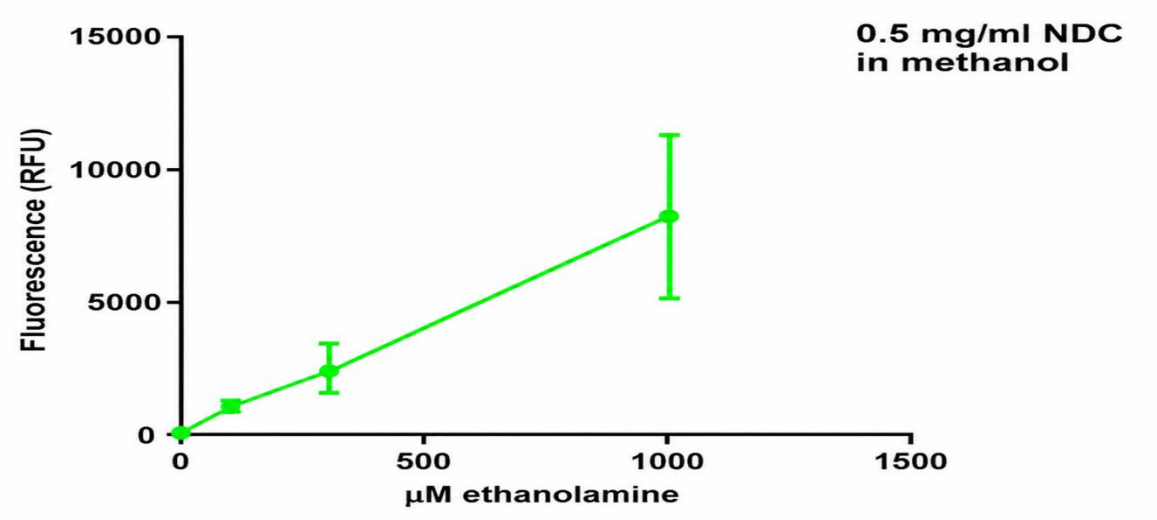


Figure 11: Effects of varying Ethanolamine concentrations on fluorescence production in the presence of 0.5 mg/ml NDC

After that, ethanolamine was tested to compare my results with Nahed Alharthi (a PhD student from the lab who graduated in 2018) her thesis result is in the next page. The concentration of NDC was 0.5 mg/ml in the presence of 2 mg/ml of sulfite (see Figure11). The limit of sensitivity was between 10 and 30 µM. The linear range was between 152 and 8400 RFU, which is low in comparison with Nahed's results, but the sensitivity had improved. In this experiment,NDC was dissolved in methanol to lower the limit of detection and, therefore, improve the sensitivity.

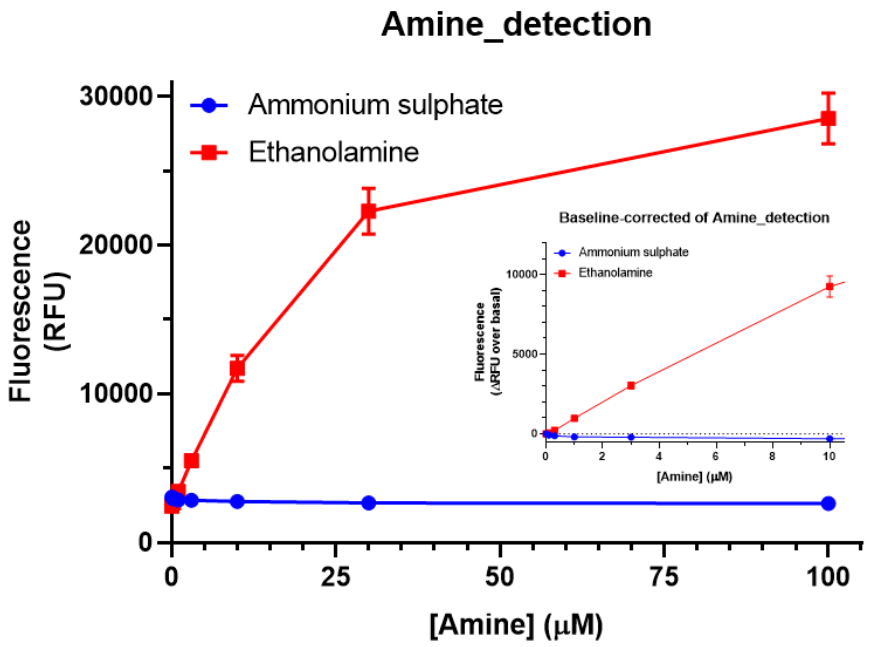


Figure 12: Effects of varying amine concentrations on fluorescence production in the presence of 1 mg/ml NDC

Nahed's result (the previous PhD student) (59),showed that the fluorescence was higher than the fluorescence of my experiment; the linear range was between 300 and 22000 RFU (figure 12). In the previous investigation, NDC was dissolved in acetonitrile to 1 mg/mL and then diluted 1:4 in 0.2 M tetraborate buffer (pH = 11.0) while in the present investigation, the NDC was diluted as 1:4 in 0.2M KPO4 buffer (pH = 11.5). However, the conditions were able to discriminate the organic amine from ammonia.

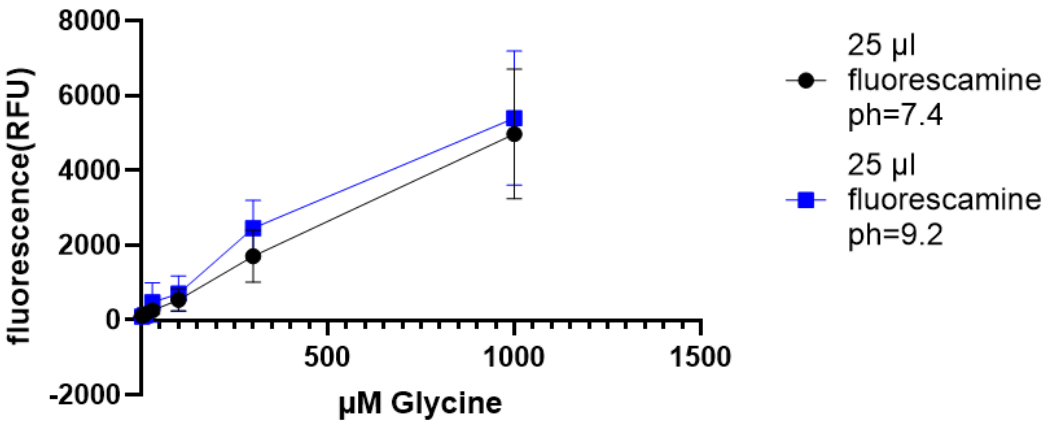


Figure 13: Effects of varying Amine concentrations on fluorescence production in the presence of fluorescamine at different PH.

Given the difficulties faced with using NDC as the derivatization reagent it was decided to use fluorescamine as an alternative to investigate if this improved sensitivity and linearity. The detection of glycine with fluorescamine as a derivatisation agent and the comparison of the different pH levels of the phosphate buffer at pH = 9.2 increased the fluorescence more than pH = 7.4. Fluorescamine increased the sensitivity (Figure 13). The LLOD was at 1 µM. The linear range was between 13 and 1797 RFU, which is low in comparison with the NDC result.

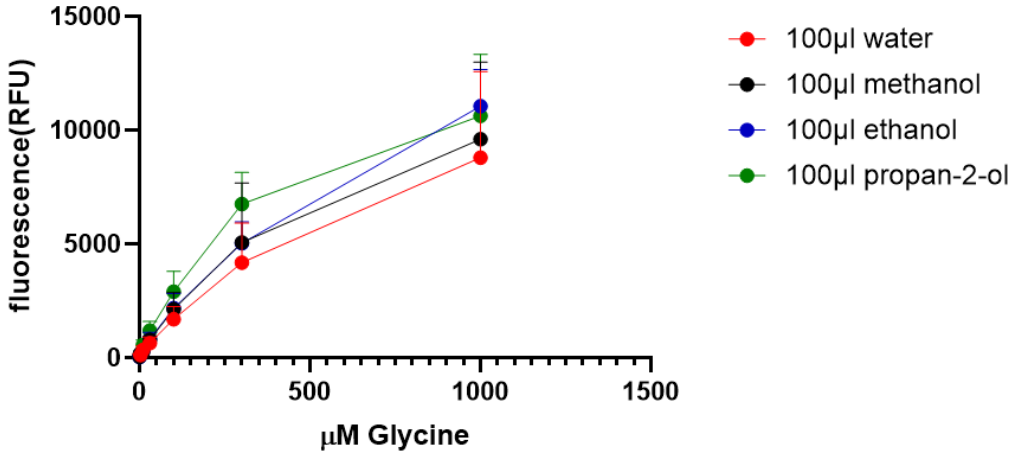


Figure 14: Effects of varying Glycine concentrations on fluorescence production in the presence of different solvents.

Adding propan-2-ol as a solvent showed by a small margin the highest fluorescence of the solvents tested. All the solvents had a LLOD of 1 µM. Propan-2-o-l produced a range of fluorescence between 66 and 10626 RFU (see figure 14)

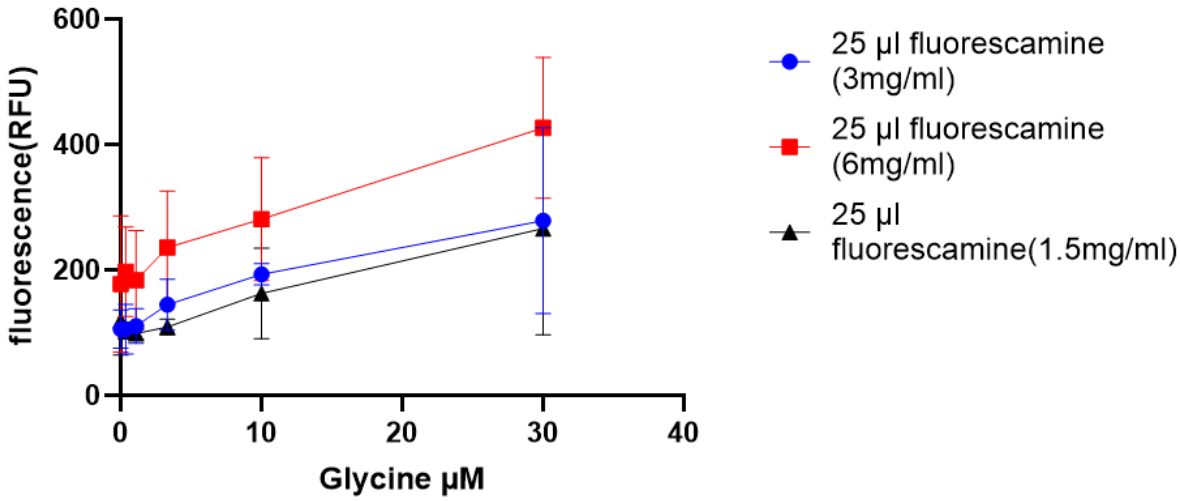


Figure15: Effects of varying glycine concentrations on fluorescence production in the presence of different concentrations of fluorescamine .

In all three concentrations, the limit of sensitivity was 30 µM (see figure 15).The blank value in 3 mg/ml was 105, in 6 mg/ml was 177 and in 1.5 mg/ml was 119 RFU.The 6 mg/ml concentration showed the highest fluorescence, but the highest blank value. The linear range of the fluorescence was 177 to 426 RFU. Fluorescamine increased the sensitivity, but it produced less fluorescence than NDC.

Table3: A summary table of the use of NDC as a derivatisation agent for glycine detection

Substrate	NDC solvent	[NDC] (mg/ml)	Phosphate buffer pH	Addition	Conc (mg/mL)	LLOD (µM)	Blank value	Linear range (RFU)
Glycine	MeOH	1	10.5			1	39	39–134
Glycine	MeOH	1	10.5	Sulfite	1	100	250	Less than 8850
Glycine	MeOH	1	10.5	Sulfite	2	100	245	350–8850
Glycine	MeOH	1	10.5	Sulfite	50	300–1000	236	Less than 8850
Glycine	MeOH	1	10.5	Sulfite	100	300–1000	41	Less than 8850
Glycine	MeOH	0.5	10.5			300–1000	427	6650–427
Glycine	MeOH	1	10.5			100–300	368	Less than 6665
Glycine	MeOH	2	10.5			100–300	375	Less than 6665
Glycine	MeOH	4	10.5			100–300	277	Less than 6650
Ethanolamine	MeOH	0.5	11.5	Sulfite	2	10–30	152	152–8400
Ethanolamine	Acetonitrile	0.5	11.5	Sulfite	2	10–30	568	576–21654
Ethanolamine	Acetonitrile	1	11.5	Sulfite	2	10–30	734	Less than 21654
Ethanolamine	Acetonitrile	2	11.5	Sulfite	2	100–300	805	Less than 21654
Ethanolamine	Acetonitrile	4	11.5	Sulfite	2	30	528	Less than 21654
Ethanolamine	Acetonitrile	4	11.5	Sulfite	2	30–100	39	Less than 21654
Ethanolamine	Acetonitrile	0.5	11.5	Copper	25	30–100	41	30 to 367

The quantification of primary amines, such as glycine and ethanolamine, is essential for measuring the activity of enzymes that hydrolyze AEA and NAAs to release their products, which are primary amines. Because these products do not naturally fluoresce, they must be derivatized using fluorogenic reagents to be detected at low concentrations.

This study used two distinct chemical substances to generate a fluorescent product: naphthalene-2,3-dicarboxaldehyde (NDC) and fluorescamine.

Table 3 summarises the results of using NDC to detect amino acids, specifically glycine. The assay was inexpensive and simple, using a common buffering agent, potassium phosphate. Ethanolamine was also used as a target, using NDC to compare the results with the previous PhD student.

The first experiment detected glycine without any addition, which can be found in the first row of the table. For the standard curve, see Table 3. The LLOD and the blank value were low, which is good, but the fluorescence was also low (see Figure8), which led to the addition of sulfite, which increased fluorescence levels. Still, the LLOD and blank values were high; 2 mg/ml of sulfite showed the lowest blank value and LLOD (see Figure9). Four concentrations of NDC were examined without adding sulfite. As a result, 0.5 mg/ml NDC gave the highest fluorescence, but the highest LLOD and blank values; 4 mg/ml NDC gave the lowest fluorescence, but the lowest LLOD and blank values, which is favourable (see Figure 10).

In ethanolamine detection, 0.5 mg/ml NDC produced the highest fluorescence and the lowest LLOD and blank values, which is favourable. The addition of 2 mg/ml of sulfite decreased the blank value but did not increase the fluorescence (see Figure11). Copper is added to the reaction to stabilize the adduct or quench the background "blank" signal. [60]The addition of copper did not increase fluorescence levels and did not decrease the LLOD or the blank values. Detection of ethanolamine using NDC gave a lower LLOD but a higher blank value than glycine detection. The main principle of an alternative to the amino acid quantification assay is that fluorescamine binds directly to the amino group of the amino acid to produce a fluorescent adduct. The table below summarises, using fluorescamine to detect glycine, taurine, alanine and serine.

Table 4: Summary table of the investigation of fluorescamine as a derivatisation agent for amino acid detection.

Substrate	[Fluorescamine] (mg/mL)	Phosphate buffer pH	Addition	LLOD (µM)	Blank value	Linear range (RFU)	Comment
Glycine	3	9.2		10–30	83	83-5397	
Glycine	3	7.4		10–30	91	91–4973	
Glycine	3	9.2	MeOH	1	41	41–9601	
Glycine	3	9.2	EtOH	1	32	32–11062	
Glycine	3	9.2	propan-2-ol	3	66	66–9986	propan-2-ol showed the highest fluorescence
Glycine	3	7.4	propan-2-ol	1	90	90–9405	
Taurine	3	7.4	propan-2-ol	1	111	111–7787	
Alanine	3	7.4	propan-2-ol	1	156	150–7787	
Serine	3	7.4	propan-2-ol	1	93	93–9604	
Glycine	3	7.4		30	105	105–278	6mg/ml concentration showed the highest fluorescence but also the highest blank value
Glycine	(1.5mg/ml)	7.4		30	177	177–426	
Glycine	(6mg/ml)	7.4		30	119	199–266	

The first test to detect glycine used 3 mg/ml fluorescamine (see Table 4), which produced a low LLOD and blank compared to the NDC. However, it also produced lower fluorescence values. The blank value using potassium phosphate buffer (pH = 9.2) produced lower blank values than pH = 7.4 buffer (see Figure 13).

The addition of methanol, ethanol, and propan-2-ol decreased the LLOD to 1-3 µM at pH = 9.2. The addition of propan-2-ol reduced the LLOD of glycine, serine, alanine and taurine to 1 µM at pH = 7.4 (see Figure 14). Increasing the concentration of fluorescamine to 6 mg/ml produced the highest fluorescence levels but also the highest blank value. Therefore, 3 mg/ml appeared to be the optimal concentration of fluorescamine (see Figure 15)

5.4 Discussion:

A fluorometric assay was developed for the measurement of glycine released during the hydrolysis of N-acyl amino acids (NAAs), and was optimised systematically by testing derivatisation reagents naphthalene-2,3-carboxaldehyde (NDC) and fluorescamine, reagent concentrations, pH, solvent and reaction conditions.

NDC is electrophilic, so it needs a nucleophile, such as sulfite or copper ions, to increase reactivity with the amine group of the amino acid and produce a cyclic fluorescent adduct. (60) A previous study using OPA (O-phthalaldehyde), which is similar in structure to NDC, to detect GABA showed that the addition of sulfite led to a more stable product than thiol or other additions. The addition of sulfite improved sensitivity, with a detection limit of approximately 1 nM. (61) Another study demonstrated that OPA detected primary amines, and the product was stable in comparison with other additions; the LLOD of tryptophan was 0.061 mM. (62) The reason for choosing NDC as a derivatisation agent was that the NDC generates a product that is more stable and has a longer wavelength than OPA.(63) However, the fluorescence of naphthalene-2,3-carboxaldehyde (NDC) was relatively weak even after optimization with sulfite. Therefore, Fluorescamine is tested to improve the fluorescence and the sensitivity. A previous study showed that Fluorescamine showed higher fluorescence signals than NDC as it reacts rapidly with primary amines under alkaline conditions to form highly fluorescent product. (58)In our study, Fluorescamine increased the sensitivity, but it did not increase the fluorescence in comparison with the NDC.

5.5 Conclusion and Future Plans:

The development assay used to detect the amino acids was limited by low sensitivity and low fluorescence. Therefore, ABPP will be employed to identify possible hydrolytic enzymes in a cell line model, and the separation will be done through SDS-polyacrylamide gels. The technique will also be applied to samples of human and rat brains. The goal is to identify which bands are sensitive to NAGly and determine their molecular size and pharmacological profile. This should allow for the identification of individual serine hydrolase enzyme activities, which can be expressed in recombinant systems to confirm the impact of NAAs on functional measures.

6 Chapter 4: Detecting Serine hydrolyzing enzyme on human brain by Activity-based protein profiling (ABPP)

6.1 Introduction:

While much of pharmacological research makes use of rodents as models, it is important to determine whether similar enzyme profiles and pharmacological sensitivities are observed in human brain. Although rodent models provide valuable insight into enzymatic reactions, species differences in enzyme expression, localization, and inhibitor sensitivity are well documented. The human brain, like the rat brain, is rich in lipid signaling pathways and expresses a diverse range of serine hydrolases involved in the metabolism of endocannabinoids and related lipid mediators. Enzymes such as FAAH, MAGL, and ABHD6 are reported to be present in human neural tissue, where they regulate levels of bioactive lipids including anandamide and 2-arachidonoylglycerol (2-AG). (64)(65) Given the structural similarity between these lipids and NAAs, it is likely that similar enzymatic systems are involved in NAA metabolism in the human brain.

Activity-based protein profiling (ABPP) was employed as a functional proteomic approach to detect active serine hydrolases in human brain tissue. As in the rat brain experiments, both fluorophosphonate-based probes and β-lactone-based probes were used to maximize enzyme coverage, enabling detection of enzymes that may not be labelled by a single probe class. (50)(53) This approach allows direct comparison of enzyme activity profiles between species.

Pharmacological inhibitors were used in competitive ABPP experiments to help enable the identification of individual enzyme activities. By examining inhibitor sensitivity profiles, it is possible to assess whether similar enzymes are active in both rat and human brain, as well as to identify potential species-specific differences.(44)

6.2 Hypothesis and Aim:

There are currently only a few enzymes discovered to hydrolyse NAAs, and most of the studies on human tissues are limited. The hypothesis is that NAGly is metabolized by serine hydrolases since it has a similar structure to anandamide, suggesting that FAAH would be an enzyme that could metabolize NAGly and indeed has been reported to metabolize NAGly. Also, FAAH and potentially other enzymes can hydrolyse NAGly.

Objectives:

1-to identify enzyme activities sensitive to the presence of NAGly using activity-based protein profiling of human brain tissue.
2- to make use of selective inhibitors to aid in the identification of serine hydrolases in human brain samples

6.3 Results :

In this study 6 particulate fractions of human post mortem brain prefrontal cortex tissue (The molecular marker was the ECL plex fluorescent marker, full range (RPN851E).In this blinded study, the human samples were prepared by Dr.Alexander and Dr Wafa Hourani and Dr Qulayl Aldossari conducted the reaction with inhibitors and the probe and the ABPP was performed by them. So as to avoid bias during the analysis, the identity of the conditions (inhibitors and controls) was not revealed until after the analysis was concluded. (see figure 16).

A first step, was to calculate the mean, standard deviation, and the coefficient variation of the RF values. An intention was that the coefficient of variation of the RF values should not exceed 5 %. This preliminary step provides confidence that the same band/enzyme activity is being compared across multiple gels. After the scans were completely analyzed using ImageLab and the calculations completed, the names of the inhibitors were revealed (see tables 5 in the appendix , 6, and 7).

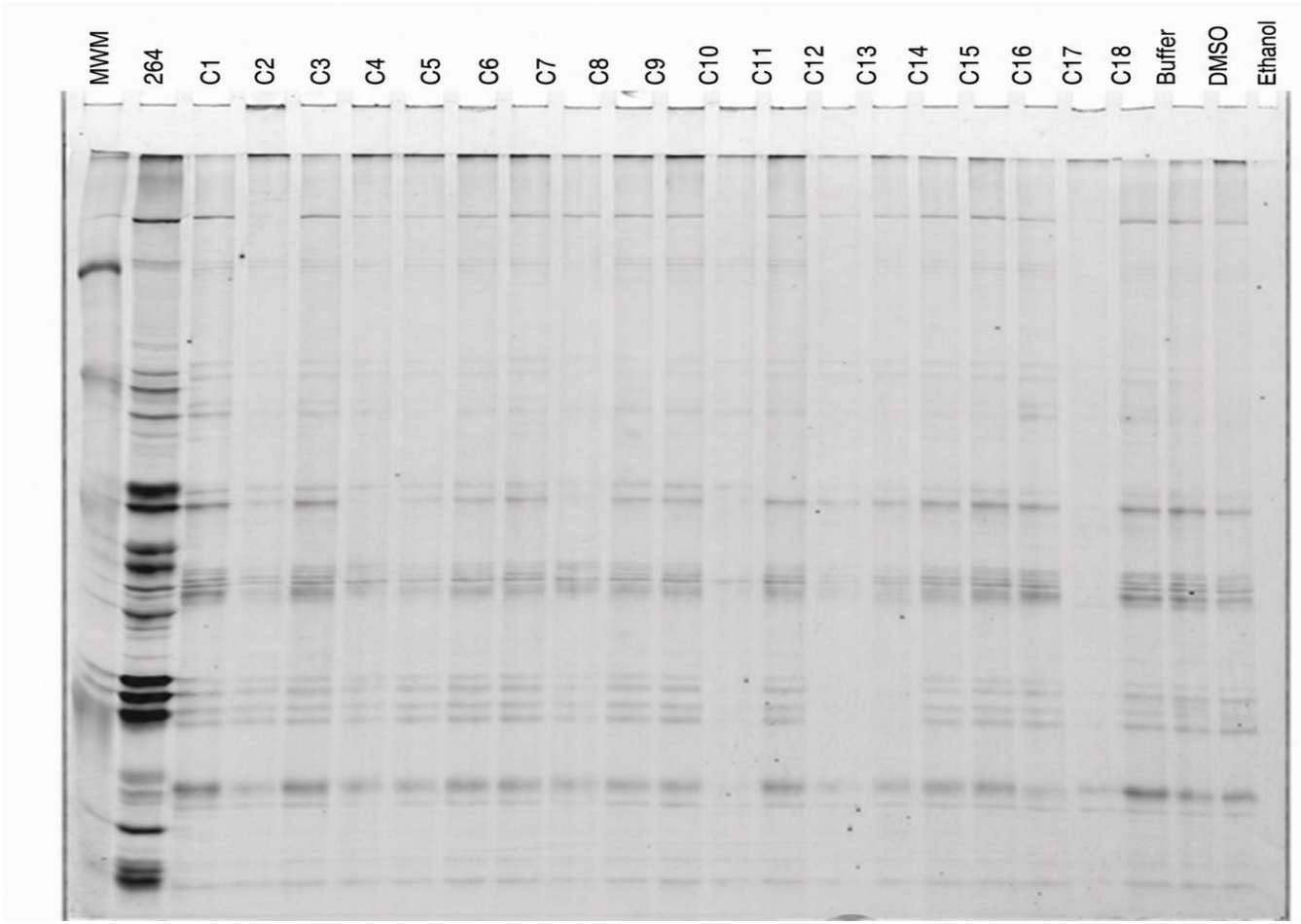


Figure 16: human brain particulate fraction, representative gel scan (Cy3 CHANNEL) MEASURED using the activity-based probe FP-rhodamine and MB064 in the presence of NAGly and other inhibitors.

Table 6: summary table of the inhibitors and their targets.

2OG	ENDOGENOUS MAGL SUBSTRATE
PEA	Endogenous FAAH substrate
2AG	Endogenous MAGL substrate
AEA	Endogenous FAAH substrate
ODA	Endogenous FAAH substrate
NAGLY	Endogenous metabolite
BMP	Endogenous metabolite
MUH	Synthetic esterase substrate
AAMC	Synthetic amidase substrate
7HCA	Synthetic esterase substrate
4NPB	Synthetic esterase substrate
MAFP	Broad spectrum SH inhibitor
URB597	FAAH inhibitor
JZL184	MAGL inhibitor
JJKK048	MAGL inhibitor
WWL70	ABHD6 inhibitor
KT203	ABHD6 inhibitor
DMSO	Solvent
ETHANOL	Solvent

Table 7: The effect of selected inhibitors or alternative substrates on serine hydrolase activities in human brain particulate fractions

Protein (kDa)	2OG	PEA	2AG	AEA	ODA	MUH	AAMC	7HCA	NPB	NAGly
>200	100 ± 11	43 ± 13	89 ± 10	31 ± 10	36 ± 6	36 ± 8	41 ± 7	63 ± 7	45 ± 8	44 ± 3
165.1	84 ± 19	62 ± 7	67 ± 16	45 ± 14	66 ± 16	72 ± 21	68 ± 20	56 ± 7	74 ± 16	74 ± 20
88	107 ± 11	52 ± 11	106 ± 12	39 ± 6	56 ± 9	56 ± 4	57 ± 8	74 ± 11	59 ± 9	55 ± 5
83.4	109 ± 13	48 ± 8	92 ± 8	36 ± 7	49 ± 8	51 ± 3	63 ± 7	71 ± 15	48 ± 5	56 ± 2
75.9	95 ± 16	24 ± 5	32 ± 9	20 ± 3	20 ± 6	21 ± 4	25 ± 7	33 ± 5	30 ± 12	22 ± 5
73.3	93 ± 11	50 ± 5	43 ± 11	49 ± 3	58 ± 9	56 ± 4	62 ± 4	44 ± 6	46 ± 11	64 ± 14
68.3	66 ± 18	40 ± 15	37 ± 9	32 ± 6	36 ± 8	29 ± 8	67 ± 22	25 ± 6	56 ± 23	37 ± 12
58.7	123 ± 17	95 ± 9	98 ± 11	68 ± 5	85 ± 13	96 ± 8	98 ± 11	87 ± 8	101 ± 7	105 ± 9
56.3	108 ± 9	45 ± 10	87 ± 14	5 ± 2	29 ± 5	38 ± 6	44 ± 6	18 ± 3	44 ± 5	47 ± 5
47.3	100 ± 10	51 ± 2	73 ± 7	47 ± 11	52 ± 3	53 ± 7	50 ± 7	90 ± 13	44 ± 7	46 ± 7
46.4	101 ± 20	65 ± 10	91 ± 20	69 ± 23	56 ± 6	67 ± 12	62 ± 14	85 ± 19	47 ± 11	55 ± 12
45.6	94 ± 11	71 ± 7	93 ± 11	65 ± 13	74 ± 8	69 ± 5	69 ± 11	101 ± 18	61 ± 11	73 ± 12
44.6	161 ± 45	60 ± 18	102 ± 29	31 ± 12	53 ± 6	69 ± 12	57 ± 8	61 ± 15	55 ± 9	61 ± 8
42	71 ± 26	76 ± 44	91 ± 50	31 ± 11	70 ± 35	57 ± 19	61 ± 16	27 ± 16	63 ± 32	43 ± 14
35.5	161 ± 23	164 ± 17	171 ± 62	116 ± 12	126 ± 28	140 ± 14	132 ± 19	127 ± 44	170 ± 44	169 ± 49
34.2	104 ± 15	107 ± 16	91 ± 12	67 ± 9	112 ± 13	110 ± 16	107 ± 14	76 ± 15	94 ± 9	99 ± 9
32.3	96 ± 20	98 ± 14	79 ± 13	70 ± 15	104 ± 19	110 ± 19	102 ± 20	55 ± 14	87 ± 18	89 ± 18
31	98 ± 10	107 ± 10	94 ± 9	67 ± 4	107 ± 9	109 ± 10	106 ± 10	76 ± 11	95 ± 11	99 ± 7
26.1	94 ± 13	54 ± 4	64 ± 8	48 ± 6	52 ± 5	54 ± 6	50 ± 7	66 ± 17	47 ± 9	50 ± 8
25.6	96 ± 14	58 ± 7	56 ± 11	53 ± 10	64 ± 13	51 ± 15	57 ± 13	44 ± 8	50 ± 8	75 ± 16

20.5	147 ± 16	92 ± 10	112 ± 32	81 ± 5	100 ± 6	109 ± 15	96 ± 9	77 ± 6	92 ± 10	103 ± 21

Protein (kDa)	MAFP	URB597	JZL184	JJKK048	WWL70	KT203	BMP	MAFP	Buffer	DMSO	Ethanol
>200	5 ± 4	50 ± 11	70 ± 11	74 ± 11	80 ± 12	74 ± 9	48 ± 8	3 ± 2	100 ± 0	81 ± 7	56 ± 10
165.1	42 ± 12	96 ± 29	51 ± 12	43 ± 14	62 ± 12	62 ± 12	81 ± 15	5 ± 2	100 ± 0	68 ± 16	77 ± 23
88	8 ± 3	59 ± 7	84 ± 10	85 ± 6	103 ± 9	74 ± 5	84 ± 7	14 ± 3	100 ± 0	106 ± 14	48 ± 13
83.4	10 ± 5	60 ± 5	91 ± 8	98 ± 13	106 ± 10	77 ± 8	101 ± 16	14 ± 4	100 ± 0	118 ± 17	56 ± 15
75.9	17 ± 9	29 ± 8	22 ± 7	38 ± 5	31 ± 6	30 ± 4	107 ± 6	18 ± 4	100 ± 0	30 ± 6	24 ± 8
73.3	48 ± 5	58 ± 9	30 ± 7	37 ± 8	32 ± 7	12 ± 2	82 ± 8	9 ± 2	100 ± 0	42 ± 3	65 ± 9
68.3	1 ± 0	30 ± 7	29 ± 7	29 ± 9	34 ± 7	33 ± 11	54 ± 10	39 ± 9	100 ± 0	27 ± 7	57 ± 14
58.7	48 ± 12	7 ± 3	77 ± 9	84 ± 11	87 ± 7	76 ± 8	60 ± 5	10 ± 5	100 ± 0	90 ± 5	83 ± 13
56.3	6 ± 3	59 ± 5	69 ± 15	77 ± 13	89 ± 10	82 ± 6	67 ± 7	10 ± 4	100 ± 0	83 ± 5	46 ± 6
47.3	8 ± 3	56 ± 8	55 ± 7	63 ± 7	69 ± 7	71 ± 7	80 ± 9	2 ± 1	100 ± 0	73 ± 5	37 ± 9
46.4	37 ± 15	63 ± 14	66 ± 11	66 ± 7	89 ± 14	81 ± 15	71 ± 14	1 ± 0	100 ± 0	79 ± 13	129 ± 50
45.6	10 ± 2	72 ± 11	83 ± 15	81 ± 11	88 ± 13	91 ± 12	81 ± 8	7 ± 3	100 ± 0	99 ± 6	91 ± 19
44.6	13 ± 6	61 ± 7	83 ± 29	87 ± 25	87 ± 20	77 ± 9	105 ± 28	9 ± 2	100 ± 0	81 ± 16	47 ± 19
42	24 ± 15	77 ± 36	69 ± 28	81 ± 25	102 ± 35	80 ± 27	112 ± 40	8 ± 26	100 ± 0	123 ± 46	63 ± 41
35.5	57 ± 37	174 ± 59	22 ± 11	9 ± 2	135 ± 19	95 ± 19	93 ± 24	9 ± 5	100 ± 0	204 ± 85	172 ± 46
34.2	17 ± 8	106 ± 15	10 ± 3	13 ± 4	86 ± 12	71 ± 9	79 ± 10	3 ± 1	100 ± 0	107 ± 6	99 ± 7
32.3	26 ± 9	96 ± 15	45 ± 13	56 ± 17	82 ± 20	68 ± 19	49 ± 10	31 ± 11	100 ± 0	144 ± 37	121 ± 32
31	34 ± 12	104 ± 11	11 ± 3	10 ± 4	102 ± 7	93 ± 9	79 ± 8	8 ± 3	100 ± 0	102 ± 4	84 ± 18
26.1	12 ± 6	69 ± 13	54 ± 9	63 ± 9	66 ± 6	55 ± 7	49 ± 8	34 ± 6	100 ± 0	74 ± 7	70 ± 7
25.6	14 ± 7	64 ± 13	50 ± 14	72 ± 12	83 ± 7	67 ± 15	62 ± 16	41 ± 5	100 ± 0	84 ± 18	86 ± 23
20.5	58 ± 18	96 ± 10	60 ± 9	82 ± 2	92 ± 4	65 ± 13	82 ± 7	11 ± 5	100 ± 0	94 ± 11	68 ± 22

Data are means ± SEM of six independent sources of human brain particulate fraction enzyme activities normalized to the buffer control . *P<0.05 Two-way ANOVA repeated measures comparison with buffer control with post-hoc Dunnett's multiple comparison.

The highlighted cells represent the reduced activity band.

6.4 Discussion :

NAAs have been described as inhibitors of FAAH, since they inhibit the binding of AEA to the FAAH.(2) A study on FAAH activities in human, mouse, and rat brains examined the inhibitory effects of NAGly, Ala, Val, Leu, Ile, Glu, Gln, and Asp on FAAH.(33)Only NAGly, Ile and Ala inhibited FAAH activity. However, this inhibition was species dependent. NAGly inhibition of human FAAH was weaker, unlike the mice and rat FAAH, while NAraAla inhibition of human FAAH was higher than mice FAAH.(33)

A study on RAW 264.7 murine monocyte cells showed that 10 μM NAGly caused a 50% increase in basal concentrations of AEA. Oral administration of NAGly to rats elevated circulating blood levels of AEA. Therefore, there is a possiblity that NAGly inhibits the FAAH which leads to the inhibiting the AEA hydrolysis, that explains the increased level of Anandamide, but this inhibition is not fully explained and examined.(66)

To test this, ABPP was employed to identify the enzymes affected by NAGly, especially the FAAH enzyme. In this study, activity-based protein profiling (ABPP) combined with pharmacological inhibitors was used to detect and identify active serine hydrolases in human brain particulate fractions(see figure 16). This method allowed enzyme activities to be assessed, the enzyme identity is based on molecular weight and inhibitor sensitivity.

Band 8 has a molecular size of approximately 58.7 kDa, so band 8 possibly corresponds to fatty acid amide hydrolase (FAAH) (see table 7). This is supported by the reduced band intensity following treatment with the selective FAAH inhibitor URB597. Previous studies have consistently shown that FAAH migrates at approximately 60–63 kDa and is selectively inhibited by URB597 in human brain tissue, supporting this assumption.(67) Functionally, FAAH is widely expressed in neuronal tissues and plays a key role in the hydrolysis of fatty acid amides such as anandamide, highlighting its importance in lipid signaling pathways.(68)

Bands 16 and 18 showed sensitivities to JJKK048, a selective MAGL inhibitor, and their molecular weights are consistent with previous reports, indicating that MAGL migrates at approximately 33 kDa in ABPP experiments.(46) (64)

On the other hand, some bands were more difficult to identify with certainty. Band 17 has a molecular size of about 34 kDa, which could mean that it is ABHD6 based on molecular weight alone. However, band 17 intensity did not decrease in the presence of the selective inhibitor KT203.

Band 5 migrates at approximately 73–75 kDa, which may correspond to diacylglycerol lipase β (DAGLβ). DH376 could be used to test the involvement of DAGLB Berger 2023.(69)

Although ABHD17A has a molecular size of 34.1 kDa, which is similar to the molecular size of band19, in this experiment, the selective inhibitor ABD957 was not investigated, so we cannot confirm that band 17 is ABHD17A. Palmostatin B inhibits a number of serine hydrolases, including ABHD17A, APT1, and APT2, and might be a useful inhibitor to screen.(70)

Band 20 migrates approximately 25.6 kDa, which may correspond to Lysophospholipase A1 (LYPLA1/APT1). This is consistent with previous studies demonstrating that LYPLA1 migrates at approximately 25 kDa and is involved in lipid hydrolysis pathways.(71) ML348, a LYPLA1 selective inhibitor, was not added in this experiment, so we cannot confirm the identity of band 20.(72)

In this study, NAGly did not inhibit bands which may correspond to FAAH, MAGL, and ABHD6, but it inhibited bands 1, 5, 7, 9, 10, 14. To confirm the identity of these bands in the future, we need to add narrower inhibitors.

Although activity-based protein profiling (ABPP) is a powerful technique for assessing the functional activity of serine hydrolases in complex biological samples, several limitations should be considered when interpreting the present findings. First, ABPP detects enzyme activity, not protein identity and thus, the enzyme identities assigned in this study were based on molecular weight and inhibitor sensitivity and are tentative without confirmation by complementary techniques such as liquid chromatography tandem mass spectrometry (LCMS/MS). Furthermore, not all serine hydrolases are labelled with equal efficiency by the FP-TAMRA probe and some enzymes may have poor probe accessibility or affinity, leading to weak or undetectable signals. Moreover, the increased complexity of human brain tissue raises the possibility of overlapping bands of similar molecular weight, making definitive identification more challenging. Further confirmation of the identities of enzymes identified by ABPP will require additional approaches such as LC–MS/MS or immunoblotting.

6.5 Conclusion and future work:

In conclusion, ABPP was successfully used to detect and study active serine hydrolases in human brain tissue. Several bands were observed and could be tentatively assigned to endocannabinoid-metabolizing enzymes, such as FAAH and MAGL, based on molecular weight and inhibitor sensitivity. These findings are consistent with what is known about endocannabinoid-metabolizing enzymes in the brain. The use of

pharmacological inhibitors was useful in supporting enzyme identification, especially for MAGL and FAAH.

However, NAGly did not inhibit FAAH, MAGL, or ABHD6, and we need to know the identity of the bands whose activity is reduced in the presence of NAGly.

using a wider range of selective inhibitors would help identify enzyme activities with more confidence, especially those whose activity is decreased in the presence of NAGly. A band which might be DAGLβ is the only enzyme activity that is reduced in the presence of NAGly.

In general, this study shows that ABPP is a useful method to look at enzyme activity in complicated tissues like the brain. However, more work needs to be done to identify the enzyme activities more precisely, such as proteomics, and to fully understand their role.

7 Chapter 5: Detecting Serine hydrolyzing enzyme on rat brain by Activity-based protein profiling (ABPP)

7.1 Introduction:

Serine hydrolases are considered one of the largest and most complicated enzyme classes in the mammalian proteome, playing an essential role in the regulation of bioactive lipids. In the central nervous system, these enzymes are the primary regulator of the Endocannabinoid System (ECS), responsible for the rapid degradation of signaling lipids such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The metabolic control of these endocannabinoids is important; uncontrolled regulation will lead to neurological and inflammatory disorders.(46)(64)Beyond the classical endocannabinoids (NAAs), particularly (NAGly), a newly discovered endogenous signaling molecule. NAGly is structurally consistent of an arachidonic acid chain conjugated to a glycine amino acid, which exists in the structure of AEA. (1)(2)Current research suggests that NAGly have an effect on the activity of the ECS, not by binding directly to cannabinoid receptors(73) , but by competing for the metabolic enzymes that degrade endocannabinoids.(66) Previous studies in rodent models have shown that NAGly administration leads to increased AEA levels. This is due to the competitive inhibition of Fatty Acid Amide Hydrolase (FAAH).(66) However, the enzymes that regulate

NAGly metabolism are still unclear, and the ability to interact and inhibit Serine hydrolases like MAGL or the ABHD family remains unclear.

Rat brain tissue was selected as a model system because it is highly enriched in lipid signaling pathways and expresses a wide range of active serine hydrolases, including an important hydrolyzing enzyme of the endocannabinoid system[64,65]. Because the majority of current research regarding NAA metabolism is restricted to rodent tissues, testing rat brain particulate fractions allows for a direct comparison with existing results. To identify the serine hydrolase enzyme activity. This study used rat brain particulate fractions. Rat tissue is a highly enriched source of membrane-bound serine hydrolases, providing an ideal physiologically environment to study the metabolic pathway of NAGly.

Traditional biochemical assays often fail to identify enzyme activity in a native proteome. To overcome this issue, Activity-Based Protein Profiling (ABPP) is performed in this study. This technique utilizes active-site directed fluorescent probes, such as fluorophosphonate-rhodamine (FP-R), which covalently bind only to the catalytic active site of serine hydrolases.

7.2 Hypothesis and aims:

There are currently few discovered enzymes that hydrolyze NAAs, and most of the studies on rat tissue are limited. The hypothesis is that NAGly is metabolized by serine hydrolases in the rat brain particulate fraction since it has a similar structure to anandamide, suggesting that FAAH would be an enzyme that could metabolize NAGly and indeed has been reported to metabolize NAGly. Also, FAAH and potentially other enzymes can hydrolyze NAGly.

The metabolic pathway of (NAAs) remains largely unclear, with current literature primarily restricted to rodent models and a limited identification of Seine hydrolases. Given the structural similarity between (NAGly) and the endocannabinoid anandamide (AEA), it is hypothesized that NAGly is metabolized by serine hydrolases within the rat brain's particulate fraction.

7.3 Objectives:

1. To identify the enzyme where the activity is reduced in rat brain tissue in the presence of NAGly by activity-based protein profiling.

7.4 Results:

In this study, six particulate fractions of rat brain tissue were investigated using FP-rhodamine to define the serine hydrolase activities. In this blinded study, the rat brain samples and reactions with inhibitors and the probe were conducted by another

researcher (Dr Alexander), so as not to be biased during the analysis, and the ABPP was performed by lab colleagues.

Each tissue fraction was incubated with FPR and 11 compounds: Two controls and nine inhibitors. Each gel had 22 samples from two donor animals with MW markers and BSA (see Figure17).A first step, was to calculate the mean, standard deviation, and the coefficient variation of the RF values. An intention was that the coefficient of variation of the RF values should not exceed 5 %. This preliminary step provides confidence that the same band/enzyme activity is being compared across multiple gels. After the scans were completely analyzed using ImageLab and the calculations completed, the names of the inhibitors were revealed (see Table 8 in the Appendix ,9 and 10)

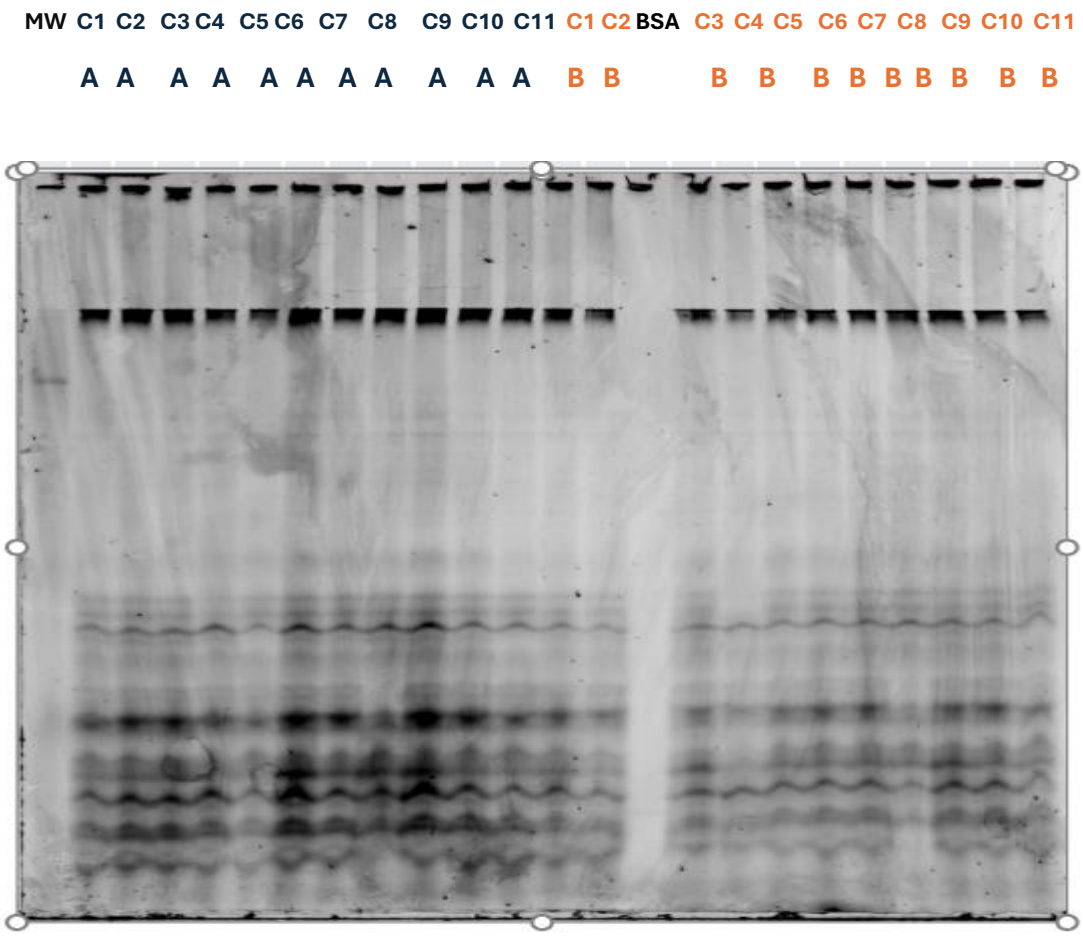


Figure 17 :Rat brain tissue ,RAT brain particulate ,from two animals (#A and #B) following treatment with compounds C1–C11. MWM, molecular weight marker.representative gel scan (Cy3 CHANNEL) MEASURED using the activity-based probe FP-rhodamine and MB064 in the presence of NAGly and other inhibitors.

Table9 : Pharmacological inhibitors used in the rat brain membrane ABPP experiments and their primary molecular targets. Compounds 1–11 were used in the rat brain membrane ABPP experiments at a final concentration of 10 μM, except Compound 8, which was tested at 1 μM because of its higher potency.

1	URB597	FAAH inhibitor
2	JNJ1661010	FAAH inhibitor
3	Control	
4	MAFP	Broad spectrum SH inhibitor
5	Benzil	CES inhibitor
6	Orlistat	DAGL inhibitor, with activity at other targets
7	JZL184	MAGL inhibitor
8	JJKK048	MAGL inhibitor
9	Control	
10	WWL70	ABHD6 inhibitor
11	KT203	ABHD6 inhibitor

Table 10: Effect of selected inhibitors on serine hydrolase activities in rat brain particulate fractions.

Molecular Size (kDa)	URB597	JNJ1661010	Control 1	MAFP	Benzil	Orlistat	JZL184	JJKK048	Control 2	WWL70	KT203
137	94 ± 10	93 ± 4	100	53 ± 3*	91 ± 14	110 ± 9	104 ± 17	115 ± 22	112 ± 18	107 ± 15	69 ± 15
76	77 ± 29	73 ± 33	100	81 ± 24	151 ± 69	118 ± 43	187 ± 106	118 ± 28	91 ± 43	170 ± 52	56 ± 30
63	124 ± 84	90 ± 51	100	96 ± 41	455 ± 285	497 ± 320	167 ± 73	1231 ± 1102	184 ± 81	173 ± 46	105 ± 75
45	45 ± 34	79 ± 16	100	59 ± 18	353 ± 188	101 ± 28	270 ± 201	112 ± 37	67 ± 23	85 ± 14	83 ± 30
41	125 ± 53	140 ± 77	100	15 ± 8	229 ± 121	175 ± 73	273 ± 145	200 ± 89	187 ± 127	124 ± 50	67 ± 18
40	204 ± 133	106 ± 45	100	88 ± 15	136 ± 35	165 ± 46	397 ± 221	328 ± 214	264 ± 180	121 ± 67	60 ± 24
37	96 ± 47	88 ± 24	100	130 ± 22	94 ± 23	164 ± 38	206 ± 82	163 ± 59	136 ± 41	114 ± 35	69 ± 16
32	100 ± 42	165 ± 98	100	47 ± 12	71 ± 32	145 ± 59	312 ± 181	58 ± 56	123 ± 53	103 ± 62	151 ± 60
30	2797 ± 2067	6805 ± 5737	100	904 ± 785	284 ± 194	5975 ± 5443	812 ± 636	6436 ± 5410	1116 ± 977	4017 ± 2924	915 ± 764
29	86 ± 29	97 ± 12	100	47 ± 8*	93 ± 10	79 ± 16	151 ± 57	54 ± 8	85 ± 18	64 ± 20	53 ± 16
26	308 ± 266	254 ± 174	100	62 ± 19	319 ± 224	312 ± 185	694 ± 397	450 ± 314	541 ± 400	108 ± 36	99 ± 36
25	40 ± 12	104 ± 49	100	68 ± 27	143 ± 69	66 ± 9	185 ± 61	150 ± 63	120 ± 67	84 ± 12	56 ± 16
23	81 ± 13	131 ± 45	100	108 ± 8	255 ± 167	206 ± 139	326 ± 175	128 ± 68	175 ± 101	96 ± 28	53 ± 7
21	111 ± 25	204 ± 72	100	153 ± 74	1850 ± 1665	1721 ± 1172	1681 ± 1460	539 ± 376	1478 ± 1336	1049 ± 878	228 ± 114
20	71 ± 20	235 ± 125	100	63 ± 15	81 ± 12	138 ± 69	293 ± 170	305 ± 245	78 ± 29	175 ± 88	40 ± 14
19	92 ± 23	193 ± 42	100	78 ± 6	143 ± 53	156 ± 74	420 ± 266	44 ± 12	173 ± 77	224 ± 101	82 ± 23

Data are presented as mean ± SEM from six independent rat brain particulate fraction preparations and are expressed relative to the control condition (Compound 3). Statistical analysis was performed using two-way repeated-measures ANOVA followed by Dunnett's multiple comparisons test. $P < 0.05$ versus Compound 3 (control).

7.5 Discussion:

In an investigation conducted by a previous PhD student's unpublished thesis using FP-rhodamine-based ABPP in rat brain soluble and particulate fractions several active serine hydrolases were identified.(74) In that study, a band (band 8) was observed at approximately 63 kDa, consistent with the reported molecular weight of FAAH. FAAH is typically detected at ~60–63 kDa and is known to be a membrane-associated enzyme involved in the hydrolysis of fatty acid amides such as anandamide.(68) However, the activity of this band was not affected by either URB597, a selective FAAH inhibitor, or MAFP, a broad-spectrum serine hydrolase inhibitor. Since URB597 is widely reported to potently inhibit FAAH activity (75), the lack of inhibition suggests that the identity of band 8 as FAAH could not be confirmed.

In the same study, bands 17 and 18 in the particulate fraction were observed at approximately 33 kDa (see table 10), which matches the reported molecular weight of MAGL. MAGL is a ~33 kDa serine hydrolase responsible for the majority of 2-AG hydrolysis in the brain. (64) The activities of these bands were reduced in the presence of JJKK048, a selective MAGL inhibitor, supporting their identification as MAGL isoforms. This is consistent with previous ABPP studies showing that selective MAGL inhibitors reduce labelling of proteins in this molecular weight range.(64)

In addition, band 19, observed at approximately 30 kDa, was proposed to correspond to ABHD6. ABHD6 is a membrane-associated serine hydrolase that contributes to a smaller but functionally important portion of 2-AG hydrolysis in the brain.(46) The reduction in activity of this band following treatment with KT203, a selective ABHD6 inhibitor, supports this assumption.

In contrast, the results obtained in the present study show some differences. Fewer bands were identified in total. Band 8 in the present study was detected at approximately 32 kDa, closer to the reported molecular weight of MAGL rather than FAAH. A reduction in activity was observed in the presence of MAFP, which is a non-selective serine hydrolase inhibitor known to target multiple enzymes. Therefore, although the molecular weight suggests that this band could correspond to MAGL, the lack of inhibition in the presence of a selective inhibitor prevents definitive confirmation. Therefore, further selective inhibitors or complementary approaches are required for confident enzyme identification. NAGly did not inhibit any band activity in our study, especially FAAH, MAGL, and ABHD6.

The activity-based protein profiling (ABPP) approach has been a useful method to probe active serine hydrolases in rat brain tissue, but some limitations should be considered for the interpretation of these data. The absence of significant inhibition by N-arachidonoyl glycine (NAGly) or the panel of pharmacologic inhibitors does not preclude the involvement of serine hydrolases in NAA metabolism. The efficiency of ABPP

depends on the ability of the activity-based probe to bind and label the catalytic site of active enzymes. Inefficient detection of poorly expressed enzymes, with low affinity for the probe, or those that do not bind well may occur. Individual bands were difficult to identify in this study because enzyme identification was based on molecular weight and inhibitor sensitivity rather than direct protein identification. In addition, NAGly might be metabolized by enzymes outside the serine hydrolase family or by multiple redundant pathways that are invisible to the fluorophosphonate probe . In addition, experimental conditions like protein preparation, enzyme stability, probe concentration and incubation conditions could have influenced the probability of enzyme labelling and inhibitor competition. Thus, while ABPP enabled the functional profiling of active serine hydrolases in rat brain tissue, definitive identification of the enzymes involved in N-acyl amino acid metabolism will require additional approaches including liquid chromatography–tandem mass spectrometry (LC–MS/MS), recombinant enzyme assays, or genetic approaches.

7.6 Conclusion and future work:

In summary, the overall band pattern matched there were fewer bands compared to the previous study; however, we could not confirm the identity of the enzyme because no enzyme activity reduction in the presence of the pharmacological inhibitor.Some bands did not clearly respond to selective inhibitors, and in some cases, only non-selective inhibitors were used, which makes it harder to draw conclusions. This shows that molecular weight alone isn't enough to tell enzymes identity.

When human brain results were compared to those from rat brains, a similar overall pattern of enzyme activity was seen. This suggests that these systems are mostly the same across species.

There are also some technical issues to think about. The bands would probably be sharper and the molecular weight guess would be more accurate if the gel preparation and separation quality were better. Also, using a bigger range of selective pharmacological inhibitors would help to make enzyme identification easier and less unclear.

Overall, these findings show that ABPP is a good way to look at serine hydrolase activity in rat brain tissue. Also, the results show that we need better experimental conditions

and other techniques, like mass spectrometry, to make sure we know what enzyme it is.

8 Overall discussion and conclusion:

8.1 Overall discussion :

This study aimed to improve our understanding of N-acyl amino acid (NAA) metabolism, with special focus on N-arachidonoyl glycine (NAGly). Because NAAs share structural similarities with endocannabinoids such as anandamide, the hypothesis is that enzymes involved in endocannabinoid metabolism, particularly serine hydrolases, might also responsiple of NAA turnover. (2),(4),(33),(39). To investigate this, two complementary techniques were used. First, a fluorometric assay was developed to detect amino acids released during NAA hydrolysis. Second, activity-based protein profiling (ABPP) was used to identify active serine hydrolases that may participate in this process.

Developing a sensitive fluorometric assay proved to be more challenging than expected. Both NDC and fluorescamine were capable of detecting amino acids following derivatisation, and several experimental parameters, including reagent concentration, pH, solvent and nucleophilic additives, were systematically optimised. These changes improved assay performance to some extent, but neither derivatisation method produced the combination of sensitivity, low background signal and reproducibility required to reliably detect the small amounts of glycine expected from NAA hydrolysis. Similar challenges have been reported in previous analytical studies using NDC, OPA and fluorescamine, where assay performance was highly dependent on reaction conditions (58),(61),(63). Although the assay did not achieve the required sensitivity, the optimisation methods provided useful information that could be used to improve future analytical methods.

Because of these limitations, ABPP became the main experimental approach used in this project. Unlike the fluorometric assay, which measures reaction products, ABPP detects catalytically active enzymes directly. This makes it particularly useful when studying complex enzyme families such as the serine hydrolases, many of which have overlapping substrate preferences (39),(48),(57). Using both the FP-TAMRA and MB064 probes increased the range of enzymes that could be detected and allowed the activity of multiple hydrolases to be examined simultaneously.

The human brain experiments identified several enzyme activities with molecular weights and inhibitor profiles that were consistent with known endocannabinoid-metabolising enzymes. In particular, activities corresponding to FAAH, MAGL and possibly ABHD6 were identified based on both their molecular weights and their responses to selective inhibitors such as URB597, JZL184, JJKK048 and KT203. These observations agree with previous studies describing the expression and pharmacological properties of these enzymes in the human brain (42),(47),(64),(65). However, despite the structural similarity between NAGly and anandamide, NAGly

itself produced relatively little competition with probe labelling. This suggests that its interaction with these enzymes may be weaker than expected or that additional enzymes contribute to NAA metabolism.

The experiments performed on rat brain tissue gave less definite results. Although several active serine hydrolases were detected, NAGly did not consistently reduce the activity of any individual enzyme. This was somewhat unexpected because FAAH has previously been reported to hydrolyse NAGly in rodent tissues (33). One possible explanation is that the enzymes responsible for NAA metabolism are expressed at relatively low levels or may not be effectively labeled by the activity-based probes utilized in this study.Also, species differences in enzyme expression or substrate selectivity may explain why the human and rat tissues produced different results. Such differences have been reported previously for enzymes involved in lipid metabolism and should be considered when translating findings from animal models to human biology (33),(39),(47).

Taken together, the results suggest that NAA metabolism is likely to be more complex than originally anticipated. Although FAAH clearly remains an important candidate enzyme, previous studies have also implicated enzymes such as PM20D1 and PAM in the synthesis and hydrolysis of NAAs (29)(35). It is therefore likely that NAA turnover is regulated by multiple enzymes rather than a single metabolic pathway. This may also explain why no individual enzyme showed strong competition with NAGly in the present study.

Like any experimental study, this work has several limitations. The fluorometric assay did not reach the sensitivity required for routine measurement of NAA hydrolysis, while gel-based ABPP identifies candidate enzymes based primarily on molecular weight and inhibitor sensitivity rather than direct protein identification. Therefore, the identity of several bands remains unclear and should be confirmed using another approach, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS).

8.2 Overall Conclusion

The purpose of this study was to investigate if serine hydrolases play a role in the metabolism of N-acyl amino acids, with a particular focus on N-arachidonoyl glycine. Although the fluorometric assay was successfully developed and optimized, it lacked the sensitivity and reproducibility needed to accurately quantify NAA hydrolysis. Activity-based protein profiling (ABPP) proved to be a more informative method, enabling the identification of several active serine hydrolases in brain tissue. However, conclusive proof that NAGly is metabolized by the serine hydrolases studied was not achieved. These findings indicate that the metabolic pathways governing NAA turnover are probably more complex than first hypothesized and are not yet fully understood. Future studies should integrate ABPP with protein identification by mass spectrometry, more sensitive quantitative assays, and a wider search for other enzyme families to

clarify the mechanisms that control NAA metabolism. In conclusion, this work contributes to our knowledge of NAA biology, demonstrates the usefulness of ABPP as a functional proteomic tool, and provides a crucial starting point for subsequent research into enzymes involved in regulating N-acyl amino acid metabolism.

9 References:

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10Appendix:

Table 5: The effect of different compounds (C1-C21) on serine hydrolase activities in human brain particulate fractions

Protein (kDa)	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
>200	1318589 ± 1042331	178471 ± 84546	943736 ± 646756	250256 ± 128936	225637 ± 123780	449269 ± 373095	570298 ± 472971	508270 ± 308078	512261 ± 381245	508669 ± 375476
165.1	388340 ± 262330	227690 ± 139295	319530 ± 242323	83970 ± 42455	184781 ± 150139	153493 ± 117511	191551 ± 155148	117096 ± 58431	185491 ± 119965	271026 ± 232956
88	445862 ± 328798	103880 ± 43436	481850 ± 360407	98258 ± 47995	250254 ± 168489	216926 ± 149123	240217 ± 161656	264837 ± 155094	271482 ± 185099	233144 ± 167141
83.4	595675 ± 467014	202358 ± 142904	391604 ± 287610	68162 ± 27642	227174 ± 151299	205329 ± 144544	353228 ± 281157	156681 ± 65526	187430 ± 118555	260685 ± 195127
75.9	870305 ± 715319	233538 ± 181684	379973 ± 326225	64610 ± 26078	76111 ± 33435	87415 ± 39398	267520 ± 209654	281675 ± 197596	191381 ± 94229	171931 ± 104277
73.3	1015486 ± 735252	418544 ± 259567	581285 ± 476890	422281 ± 249502	376303 ± 199909	381032 ± 217076	614664 ± 428462	514195 ± 359246	529784 ± 441159	839125 ± 663723
68.3	313961 ± 252625	228368 ± 208964	172784 ± 145476	68492 ± 36679	52285 ± 23636	33221 ± 18806	309326 ± 234906	37048 ± 18111	362904 ± 328086	202857 ± 184176
58.7	1742622 ± 1439978	896325 ± 652623	1113022 ± 872621	677542 ± 515432	624909 ± 371432	925719 ± 720681	1093040 ± 828135	789749 ± 532149	1030300 ± 752614	1288854 ± 1037823

56.3	3988449 ± 3279999	812127 ± 463011	2568864 ± 1990396	82531 ± 41547	697876 ± 437832	1106341 ± 845698	1706288 ± 1393067	548550 ± 381959	1448285 ± 1110480	1347354 ± 997950
47.3	1825907 ± 1388946	814726 ± 588440	1270511 ± 987612	791770 ± 569798	895058 ± 681379	898614 ± 707698	865338 ± 635022	1789697 ± 1316505	726650 ± 525549	956340 ± 762911
46.4	1532344 ± 1250348	769852 ± 574162	1504379 ± 1239686	740774 ± 457332	595972 ± 364839	996481 ± 807956	714930 ± 518801	1657596 ± 1074330	748484 ± 583346	766186 ± 597493
45.6	1643085 ± 1000840	1221073 ± 729713	1656948 ± 1079417	1355815 ± 927022	1027682 ± 514118	1156416 ± 725187	1285244 ± 790351	2120452 ± 1574580	1137543 ± 708112	1438454 ± 910839
44.6	4409970 ± 3522036	745490 ± 431034	3703766 ± 3259584	888024 ± 755566	1853880 ± 1590962	2194510 ± 1905468	1752855 ± 1467872	534672 ± 242255	2036271 ± 1763180	2058402 ± 1740277
42	519691 ± 437786	240995 ± 106542	391623 ± 221072	362966 ± 318986	327739 ± 179546	285103 ± 167423	671654 ± 528245	143333 ± 83881	265641 ± 123021	250641 ± 137161
35.5	670593 ± 524890	416556 ± 257236	426301 ± 310947	434605 ± 318412	438040 ± 288598	460760 ± 360296	319594 ± 218075	305349 ± 195182	477572 ± 366689	485581 ± 392747
34.2	1262610 ± 868849	863377 ± 448729	906879 ± 552917	574136 ± 294043	1069587 ± 589700	1014957 ± 651176	905112 ± 506698	558528 ± 242034	972517 ± 624398	1062574 ± 718280
32.3	951744 ± 698850	602584 ± 391511	611836 ± 400044	483977 ± 280257	763487 ± 492057	753262 ± 543695	628221 ± 436781	381592 ± 186675	720738 ± 574946	826086 ± 658260
31	1379444 ± 820408	1323240 ± 670316	1272384 ± 702427	948651 ± 546888	1538843 ± 817951	1570130 ± 974201	1386559 ± 758599	879781 ± 384458	1278724 ± 704367	1544378 ± 993027
26.1	6326635 ± 4124922	3319006 ± 1780443	5209786 ± 3670194	2971516 ± 1588571	3451557 ± 1903272	3874774 ± 2483306	3477509 ± 1899356	4687506 ± 2431459	3417099 ± 1952333	3761520 ± 2124108

Protein (kDa)	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21
>200	227964 ± 221164	433869 ± 312652	438582 ± 208617	465608 ± 233452	692350 ± 434767	823368 ± 619934	474893 ± 300860	107200 ± 103778	1083515 ± 803526	754408 ± 531559	520647 ± 337975
165.1	187508 ± 150922	226214 ± 127477	106468 ± 50346	83918 ± 41238	209080 ± 155620	209937 ± 158172	239017 ± 162356	27671 ± 16388	299233 ± 177739	231490 ± 186123	369218 ± 309102
88	60341 ± 53293	212702 ± 138449	211187 ± 106374	256836 ± 151199	326290 ± 186644	283100 ± 193269	300218 ± 192539	59462 ± 43341	354182 ± 239045	269875 ± 130212	166767 ± 85199
83.4	36662 ± 20883	235936 ± 160247	313497 ± 198711	323985 ± 188046	347896 ± 206546	323161 ± 216684	412978 ± 270574	39941 ± 21117	449067 ± 332889	371200 ± 214760	228545 ± 136974
75.9	93335 ± 43705	242389 ± 168920	82668 ± 37069	247288 ± 154894	178973 ± 94760	221138 ± 156116	696670 ± 460380	82112 ± 35752	626123 ± 419706	99284 ± 40036	105418 ± 43904
73.3	518923 ± 363147	586824 ± 452368	168345 ± 72033	175995 ± 771912	164662 ± 72751	110635 ± 76022	833343 ± 591034	69615 ± 32400	843022 ± 523497	322627 ± 192120	666250 ± 429439
68.3	3929 ± 3037	52578 ± 23734	71222 ± 31702	44777 ± 225400	61017 ± 26794	105135 ± 691711	208055 ± 154174	48586 ± 18865	282860 ± 182782	39931 ± 21215	93698 ± 39240
58.7	516417 ± 330406	60802 ± 29923	487134 ± 267510	561826 ± 315482	760043 ± 506520	794512 ± 571111	554374 ± 393814	75546 ± 35667	969135 ± 719023	831978 ± 610713	635162 ± 395680
56.3	279428 ± 218370	1649297 ± 1262920	1024919 ± 462210	1360942 ± 750301	2302586 ± 1604081	2667359 ± 2070559	1679221 ± 1211131	409077 ± 282988	2947810 ± 2275798	2533026 ± 2023065	1453698 ± 1075338

47.3	199970 ± 145362	840039 ± 582391	623037 ± 336360	651659 ± 343376	960214 ± 592966	1159072 ± 831446	1459426 ± 1137698	44533 ± 28302	1568642 ± 1117347	1233435 ± 934717	777410 ± 530161
46.4	816384 ± 525321	1007231 ± 782072	503687 ± 252695	720686 ± 454721	1298400 ± 1007149	1304594 ± 1040777	935140 ± 714176	16438 ± 10646	1268889 ± 750051	1137746 ± 900587	776666 ± 517582
45.6	169662 ± 98657	1150205 ± 652027	957104 ± 398340	1149426 ± 554782	1365422 ± 706649	1615311 ± 1006660	1372224 ± 865301	213318 ± 164089	1459884 ± 868951	1664083 ± 1067234	1480392 ± 808482
44.6	315122 ± 235667	1752432 ± 1435452	1200784 ± 709932	1118861 ± 621578	2134098 ± 1611105	2859840 ± 2403531	3150634 ± 2592253	310652 ± 257698	2953843 ± 2456337	2074948 ± 1698594	1622406 ± 1320904
42	313441 ± 280264	600361 ± 432886	274562 ± 128993	816048 ± 616396	8596 84 ± 666967	604995 ± 442116	590633 ± 373060	121816 ± 64511	731651 ± 540071	765537 ± 553518	414723 ± 299912
35.5	69918 ± 34166	377644 ± 269465	31136 ± 16129	30268 ± 18177	384729 ± 272211	396741 ± 299400	353428 ± 295575	160933 ± 8177	341618 ± 245866	350858 ± 213702	463517 ± 289330
34.2	182644 ± 97040	926884 ± 514965	104943 ± 49011	88146 ± 42310	678190 ± 322243	703762 ± 406472	801062 ± 531666	20002 ± 10655	1299857 ± 920957	1365585 ± 995537	1215344 ± 870562
32.3	240134 ± 133521	605902 ± 390585	101712 ± 39837	115086 ± 47983	549546 ± 376336	603317 ± 498412	501710 ± 389324	255404 ± 215971	8102654 ± 575923	750734 ± 508501	663509 ± 389119
31	445454 ± 192352	1357635 ± 733908	135868 ± 67763	129712 ± 71690	1332965 ± 707479	1494416 ± 920458	1130703 ± 695613	181738 ± 99328	1516757 ± 926265	1422360 ± 844964	1338124 ± 808069
26.1	1023038 ± 551308	5227082 ± 2729494	2606857 ± 1153768	3166736 ± 1530985	4245673 ± 2252438	4206722 ± 2525364	3194816 ± 1716999	2459995 ± 1283046	6219388 ± 3626519	4672201 ± 2535246	4385115 ± 2360122

C1-C21 represent the different test compounds of the assay. Data are means ± SEM of data from six human brain particulate fractions.

Table 8: the effect of selected inhibitors on serine hydrolase activities in rat brain particulate fractions

Protein (approximate molecular size)	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11
137	587926 ± 102704	624286 ± 113916	672273 ± 130495	348104 ± 67524	609890 ± 137019	699768 ± 102254	627897 ± 92193	650597 ± 81705	651457 ± 84368	644208 ± 90787	417473 ± 108645
76	7451 ± 3203	7750 ± 4482	11412 ± 3781	8162 ± 3130	11241 ± 5679	8500 ± 2687	17798 ± 11171	10506 ± 2253	8099 ± 3843	11031 ± 2374	3439 ± 1972
63	4837 ± 2083	6980 ± 3273	15429 ± 6807	9870 ± 5713	25469 ± 8681	23116 ± 15393	30047 ± 16690	25289 ± 9622	28400 ± 15674	20073 ± 8925	4640 ± 2592
45	18807 ± 13221	31490 ± 10594	39154 ± 10805	22211 ± 10765	68165 ± 5513	45067 ± 15792	111667 ± 78199	41655 ± 12489	28415 ± 13387	36320 ± 11773	34086 ± 12790
41	36500 ± 8529	32526 ± 5282	53433 ± 15582	12061 ± 8038	58761 ± 10111	58611 ± 14144	107874 ± 65433	57608 ± 13645	48317 ± 18038	46718 ± 14870	49640 ± 21717
40	60763 ± 14769	50141 ± 16063	73965 ± 18101	69752 ± 26952	79269 ± 22932	101223 ± 36708	189985 ± 121773	103206 ± 30622	86835 ± 35831	56836 ± 27448	57970 ± 33969
37	51930 ± 16092	57222 ± 16501	62663 ± 13837	72443 ± 16120	60286 ± 18720	80000 ± 12301	79464 ± 8908	66474 ± 10474	63484 ± 9495	49210 ± 5451	40793 ± 9821
32	33102 ± 10502	46622 ± 15779	41382 ± 12178	17375 ± 5900	23476 ± 8431	34185 ± 10177	119432 ± 83701	7281 ± 6339	38100 ± 14329	31737 ± 16520	43508 ± 15893
30	52468 ± 14602	127017 ± 33184	88545 ± 35499	82764 ± 29115	56513 ± 25444	137007 ± 47702	164288 ± 102962	83563 ± 34320	113704 ± 44813	135988 ± 32680	70293 ± 25977

29	241566 ± 60444	300884 ± 54045	324963 ± 63285	169571 ± 56385	287912 ± 35227	269564 ± 83827	415588 ± 104501	171916 ± 35741	300828 ± 98364	214430 ± 81788	174949 ± 62099
26	51874 ± 16500	69163 ± 12726	78700 ± 26886	34483 ± 10069	76505 ± 8264	95998 ± 14612	207381 ± 102841	104327 ± 11882	107376 ± 19179	60648 ± 20423	49832 ± 14705
25	35505 ± 5138	78295 ± 15993	114472 ± 22766	59064 ± 19578	102640 ± 22513	71629 ± 15351	194170 ± 77251	122051 ± 21696	88456 ± 21011	89868 ± 15463	60259 ± 19697
23	98042 ± 17863	134272 ± 32299	140143 ± 29346	154277 ± 30142	150173 ± 13988	126423 ± 31281	283726 ± 176208	99843 ± 22583	127819 ± 30292	105788 ± 26608	75608 ± 19446
21	47092 ± 12456	90290 ± 36725	65144 ± 22000	57968 ± 16348	90309 ± 9963	153809 ± 32363	154114 ± 60266	105737 ± 44322	91302 ± 24182	95623 ± 16995	64376 ± 18490
20	46831 ± 12986	95223 ± 18418	80667 ± 23346	45314 ± 18578	53278 ± 9774	59646 ± 21706	146844 ± 80752	74427 ± 22422	60990 ± 17636	76033 ± 15962	42593 ± 16974
19	72119 ± 21254	149006 ± 36761	86442 ± 23202	63721 ± 14102	88886 ± 15918	95439 ± 27545	282565 ± 180983	33928 ± 10155	101400 ± 26899	143310 ± 44807	73948 ± 26937

Data are means ± SEM of six independent sources of rat brain particulate fraction enzyme activities.