Abstract

p600 is a large protein that is enriched in the brain and expressed in neurons. Previous work has shown a diversity of functions for p600 in several different organisms and cell types. Following a report that p600 associates with the Ca\(^{2+}\) sensor calmodulin (CaM), we hypothesized that p600 was acting as a Ca\(^{2+}\) signalling protein in hippocampal neurons, a population of cells which is susceptible to Ca\(^{2+}\)-induced death. We found that in the presence of Ca\(^{2+}\), p600 associates with CaM and the ubiquitous Ca\(^{2+}\)/CaM-dependent protein kinase IIα (CaMKIIα). This interaction is mediated by a direct and atypical interaction between residues 4086-4111 on p600 and CaM. Using a peptide based on this sequence to specifically block the p600/CaM interaction, we determined that this interaction is required for the survival of hippocampal neurons. This survival was found to be dependent on the Ca\(^{2+}\)-influx through the N-methyl-D-aspartate (NMDA) receptor during ambient culture activity. We next assessed the regulation of CaMKIIα in order to determine the mechanism by which p600 promotes neuronal survival. Under pathological conditions, namely elevated intracellular Ca\(^{2+}\) concentrations and decreased intracellular pH, CaMKIIα undergoes a rapid aggregation. We therefore used this aggregation as a marker for pathological intracellular conditions. Predictably, treatment with glutamate and glycine to induce Ca\(^{2+}\) entry caused CaMKIIα aggregation, and depletion of p600 by RNAi sensitized neurons to this effect. Within each neuron, the amount of CaMKIIα aggregation was found to correlate significantly with the amount of fragmentation of the endoplasmic reticulum (ER), suggesting that p600 controls ER morphology. Using a battery of inhibitors, the aggregation of CaMKIIα was found to depend primarily on Ca\(^{2+}\) influx through NMDA receptors, on ER Ca\(^{2+}\) stores by way of inositol-3-phosphate receptors, and somewhat on influx through L-type voltage-dependent channels. The microtubule-stabilizing compound paclitaxel decreased the likelihood
of CaMKIIα aggregation in directly-depolarized neurons, but not those given glutamate/glycine treatment, indicating that p600 regulates microtubule stability in response to certain sources of Ca²⁺ entry. In sum, these data show that p600 promotes the survival of hippocampal neurons through a novel role as a Ca²⁺-signalling protein.
Acknowledgements

Over the course of my research career I have been supported by an Achievers In Medical Science award, an Alberta Heritage Foundation for Medical Research studentship, and a Dr. T. Chen Fong doctoral scholarship. I am grateful to those who granted me these awards. I am also grateful to those who donated their time to teach me to be proficient in the laboratory. In this regard, I would particularly like to thank Dr. James Wang, Su Shim, and Dr. Gernot Neumayer for initiating me in the mysteries of molecular biology. I would also like to thank Dr. Michael Colicos, as well as his laboratory personnel, for patiently teaching me the techniques of neuronal culture and photoconductive stimulation. I would like to thank all those who have given their time to scrutinize, guide, and improve my work, especially the members of my supervisory committee, Drs. Minh Dang Nguyen, Michael Colicos, Roger Thompson and Ken Lukowiak. I would also like to thank Drs. Kathryn Todd and Jonathan Lytton for taking the time to become familiar with my work and for their helpful comments on this thesis. Most of all, I am indebted to my supervisor Dr. Minh Dang Nguyen for many years of good counsel, and for his innumerable efforts to help me to succeed.
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<th>Definition</th>
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<tr>
<td>2-APB</td>
<td>2-aminoethyl diphenylborinate</td>
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<td>2-APV</td>
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<tr>
<td>a.a.</td>
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<td>A.U.</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
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<td>phosphate buffered saline</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>paraformaldehyde</td>
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<td>protein kinase C</td>
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<td>postsynaptic density</td>
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<td>RNA interference</td>
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<td>ryanodine receptor</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>sodium dodecyl sulfate</td>
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<td>standard error of the mean</td>
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<td>UBR4</td>
<td>ubiquitin protein ligase E3 component n-recognition 4</td>
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<tr>
<td>VDCC</td>
<td>voltage-dependent Ca$^{2+}$ channel</td>
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<tr>
<td>z-VAD-fmk</td>
<td>benzyloxycarbonyl-VAD-fluoromethyl ketone</td>
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Chapter One: Introduction

Over two decades of study have satisfactorily described many functions of p600 and its homologs, but those functions remain largely or completely distinct from one another. p600 therefore has no single classification, and is studied in different contexts with respect to its various functions. The first chapter is therefore dedicated to a description of the molecule and an enumeration of its many functions. It begins with a survey of the p600 gene, its protein sequence and evolutionary conservation, and the functions of its homologs in the two organisms in which it has been studied. The various functions of p600 in the mammalian cell are then detailed, along with the phenotype of the lethal p600 knockout mouse model. Particular attention is given to the role of p600 in the brain and neurons, wherein p600 has been shown to act a microtubule-binding and endoplasmic reticulum-associated protein. Based on the lethality of p600 knockout mice, the high expression of p600 in the brain, its expression in neurons, and its Ca\(^{2+}\)-dependent ability to bind the Ca\(^{2+}\)-sensor calmodulin, p600 is hypothesized to regulate Ca\(^{2+}\)-dependent neuronal survival in neurons. The second part of the introduction provides context for this hypothesis, detailing the origins of cytotoxic Ca\(^{2+}\) elevation in the neuron, and the mechanisms of Ca\(^{2+}\)-induced neuronal death. Given the role of p600 as a microtubule-associated protein, interaction between the microtubule cytoskeleton and Ca\(^{2+}\) signalling in neurons is reviewed. Finally, the pathological aggregation of the ubiquitous hippocampal enzyme Ca\(^{2+}/\text{CaM}\)-dependent kinase II\(\alpha\) is discussed along with its utility as a pathological marker in neurons.
1.1 The p600 protein

1.1.1 The mammalian p600 gene

The p600 gene was first cloned (as KIAA0462) in a 1997 study by Seki et al. in which they reported 91 complementary DNAs (cDNAs) of supposed large brain proteins in humans [1]. A follow-up study by the same group in 2000, again screening for large human brain protein cDNAs, partially cloned p600 (as KIAA1307) amongst a total of 150 cDNAs [2]. Chromosomal mapping in both cases placed p600 on chromosome 1. The current human genome build (GRCh38) reports that p600 is encoded on the reverse strand of the first chromosome between residues 19,401,000 and 19,536,770. 106 exons are spliced into 6 predicted protein-coding transcripts, 1 of which encodes the 5183 amino acid (a.a.) protein which is the focus of this thesis ([3]; illustrated in Figure 1.1).
Figure 1.1. The human *p600* gene is predicted to encode six protein-coding transcripts. *p600* is located on the short arm of the first chromosome (a), encoded on the reverse strand between the indicated residues (b). The six predicted protein-coding transcripts are shown as pre-messenger RNA (mRNA) (red bars). The pre-mRNA encoding the canonical 5183 a.a. isoform is depicted with a bolder line. These data are from the Ensembl.org database [3].
1.1.1.1 Nomenclature

The p600 protein is variously referred to by its large molecular weight (p600 is “protein of 600 kDa”), or by one of several names derived from its various functions. “Ubiquitin protein ligase E3 component n-recogin 4” (UBR4) has become a common and perhaps the predominant name, since it has principally been studied in the context of its role in the proteosomic degradation pathway (see section 1.1.4.1). It has similarly been called “zinc finger, UBR1 type 1” (ZUBR1) for its small but distinguishing zinc-finger motif similar to those found in ubiquitin pathway proteins (more in sections 1.1.2.1 and 1.1.4.1). Another name, now obsolete, is “retinoblastoma-associated factor of 600 kDa” (RBAF600) after its association with the retinoblastoma complex (see section 1.1.6.2). Finally, it was initially identified as “microtubule-associated factor of 600 kDa” MTAF600 for its association with microtubules. Though descriptive names based on protein function are generally preferable, p600 can be excused from this generality since it is multifunctional, and because its large size does not permit much confusion from its size-based descriptor. The generic size-based descriptor is especially desirable here given the marginal significance in the chapters to follow of the functions from which it has acquired its other names. It will therefore be called p600 for the remainder of this thesis.

1.1.2 The mammalian p600 protein

The p600 protein is biochemically undistinguished. It migrates at 600 kDa in denaturing electrophoresis (e.g. Figure 2.3A), which is to be expected for any protein without egregious abnormalities. Gel filtration chromatography of the C-terminus suggests that the C-terminal region (a.a. 4480-5183) has a tendency to dimerize [4], which indicates that endogenous p600 may do the same. Beyond this, essentially nothing is known about its molecular properties.
Given the paucity of empirical evidence regarding its structure and function, structural predictions using the primary sequence of p600 are of particular value for those attempting to discern its functions. Its sequence however yields notably few hints on its functions. Domains that can be predicted with near-certainty, namely one Zn-finger domain (see the section immediately following), amount to a mere 65/5183 residues (1.3%) of the total sequence of p600, and even the function of that region is unknown. It will however be important when trying to describe a unified role of p600 to be able to localize these functions, and to see which are retained through evolution. The following 2 subsections, summarized in Figure 1.2, will serve as a basis for trying to map the functions of p600 onto its sequence.

1.1.2.1 Primary structure prediction
An alignment of the complete a.a. sequence of human p600 against the proteomes of other organisms reveals that homologs are to be found in chimpanzees, mice, and rats in an essentially identical form ([5]; see Table 1.1). Alignments against our more distant relatives shows that the C-terminal portion of p600 is more far more conserved and the N-terminus more variable (Table 1.1). Sequence alignments between human p600 and its distant homologs in D. rerio, D. melanogaster, C. elegans and A. thaliana (Table 1.1) show a consistent and distinct cut-off corresponding to residues 3387-3406 on human p600 before which there is a “linker” region. For example, the p600 homolog in D. rerio shares 91% sequence identity with human p600 on the C-terminus (after residue 3387), 84% sequence identity in the N-terminus (up to residue 3331), but no identifiable similarity in residues 3331-3387 [5]. Thus it would appear as though there is a natural distinction between the N-terminal portion (ca. 1-3330), the C-terminal portion (ca. 3390-5183), and the linker region in between (annotated on Figure 1.2). In the other organisms
assessed in Table 1.1, the portion of p600 on the N-terminal side of the linker region shows only passing sequence identity or none at all. In fact the N-terminus is apparently largely dispensable in *C. elegans*, in which all but 1000 residues are missing. Though the N-termini share little or no sequence identity between these proteins, it is worth noting that with the exception of the *C. elegans* homolog the N-termini share nearly identical size (see Table 1.1).

A more targeted search for conserved domains [6] reveals three regions of interest. The first, residues 1660-1724, contains a zinc-finger motif. These motifs, in which cysteine and histidine residues coordinate a Zn ion, were originally described as binding nucleic acids but are increasingly found in other roles [7]. Though the abundance of roles for Zn-finger motifs precludes speculation on its precise function, this motif is so closely homologous to that found in other ubiquitin-recognizing proteins as to have been named the ubiquitin-recognizing (UBR)-box ([8]; also see section 1.1.4.1). The second region of interest, residues 3660-3704, is predicted to encode a zinc ribbon motif, a variant of the Zn-finger. However this region registers only as a weak match, and the other proteins containing this motif are found only in a number of bacteria, one archaeon, and an amoeba. It is therefore probably a false-positive match, but may still denote a separate domain. The third region of interest, residues 4364-5160, is a subset of the C-terminus that is extremely well conserved between mammals (*e.g.* *M. musculus*), insects (*e.g.* *A. aegypti*, *D. ananassae*) and plants (*e.g.* *A. thaliana*, *O. sativa*, *V. vinifera*) [6].

From these sequence alignments we know that p600 is well, but not universally, conserved in eukaryotes. For example, there is no identifiable homolog of p600 in the yeast *S. cerevisiae* or in the snail *L. stagnalis* [5]. These alignments also make evident a natural distinction between the N-terminus, C-terminus, and a linker region in between.
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<thead>
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<th>Name(^1)</th>
<th>Length</th>
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<td>23352</td>
<td>UBR4</td>
<td>5183</td>
<td>100% (100%)</td>
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<tr>
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<td>UBR4</td>
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<td>69116</td>
<td>Ubr4</td>
<td>5180</td>
<td>97% (98%)</td>
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<tr>
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<tr>
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<td>407625</td>
<td>ubr4</td>
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<tr>
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<tr>
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<td>820398</td>
<td>BIG</td>
<td>5098</td>
<td>34% (52%)(^5)</td>
</tr>
</tbody>
</table>

Table 1.1. Protein sequence identities between p600 and its cognates in various model organisms. Analogs of human p600 (UBR4) were located using the NCBI’s Homologene program [9]. Sequence identities were found by running pairwise alignments in the NCBI’s BLAST tool [5] against the human sequence. Absolute identities are given as a percent match to the human sequence. The number given in parentheses is the match for similar a.a. residues. For the final 4 comparisons, the identities given are only for the invariable region (C-terminus; see section 1.2.1.1). In *D. rerio* the alignment is interrupted by a variable region and in the latter three the N-terminus is so dissimilar as to preclude meaningful alignment. \(^1\)The primary name under which it is listed in the NCBI database, \(^2\)Corresponding only to residues 3387-5183 on human p600, \(^3\)Corresponding only to residues 3392-5183 on human p600, \(^4\)Corresponding only to residues 3390-5175 on human p600, \(^5\)Corresponding only to residues 3406-5009 on human p600.
1.1.2.2 Secondary and tertiary structure prediction

Given the association of p600 with membrane fractions after biochemical fractionation [10], it is possible that p600 associates directly with membranes by way of transmembrane domains. Computational predictions indicate that a region on p600, amino acids 329-430, contains residues in an orientation conducive to the formation of transmembrane helices [11]–[14].

A coiled-coil domain is a region of a protein in which two or more α-helices form a bundle, resulting in a relatively stiff and fibrillar structure ([15] p.158). Far from being simply structural, coiled-coiled regions partake in a variety of functions including protein-protein interactions [16]. Thus locating coiled-coiled regions on p600 could help to demarcate its domain structure and possible regions of protein interaction. Coiled-coil regions are stabilized by a heptad repeat, a repeating pattern of hydrophobic residues in the primary sequence which favours the assembly of two or more helices into a coiled-coil [15]. Their presence can therefore be inferred from certain repeating heptads in the primary sequence. Two different predictions methods predict that a narrow region, residues 4749-4834, has a strong tendency to form a coiled-coil structure [16] [17]. An assembled coiled-coil would tend to stabilize adjacent residues into the same conformation, so it is quite possible that this region extends in one or both directions on the sequence.
Figure 1.2. The domain structure of human p600, predicted from its primary sequence and based on previously published work. The belated division between the N- and C- termini is inferred from the evolutionary conservation of the region denoted as the C-terminus (section 1.1.2.1). Details on how the transmembrane, Zn-finger, Zn-ribbon and coiled-coil domains were predicted are to be found in sections 1.1.2.1 and 1.1.2.2. Further details on the Zn-finger/UBR box domain are found in [18] and discussed in section 1.1.4.1. Details on the microtubule and ER-binding regions can be found in [10] and are discussed in section 1.1.10.
1.1.3 Functions of the homologs of p600

This thesis concerns the function of mouse, rat and human p600 which are assumed, based on their essentially complete sequence identity, to have identical function. Homologs or close variants of p600 are however to be found in a number of species (summarized in Table 1.1). The function of the protein product has only been studied in two non-mammalian species, the fruit fly Drosophila melanogaster and the cress Arabidopsis thaliana. Though p600 is not named as such in any of the studies in the following sections it will be called that for clarity. The actual name by which it is described in the study is given parenthetically.

1.1.3.1 CALO/pushover (D. Melanogaster)

The earliest indication of the function of p600 (as “line 3420”) is from a 1992 study in which the use of an enhancer trap revealed that p600 is transcribed in the primary olfactory organ of the antenna [19]. Though the vast difference between the olfactory organs of Drosophila and mammals makes this observation of questionable use to those not directly engaged in the study of Drosophila, the authors also noted “some staining within the central nervous system.” They also foreshadowed future studies in noting that the males from this line were sterile. A year later another group reported two p600 mutants (as “purity of essence”) amongst a list of male-sterile mutants, but were able to offer no insight as to the mechanism of this sterility [20]. A later study would somewhat correct this deficiency by showing that p600 (purity of essence) mutants show defective sperm individualization, with multiple defects in subcellular organization [21]. This study also assessed viability and observed that the homozygous adults of the p600 mutant were found in predicted Mendelian abundance [21]. One would of course require an actual knockout
rather than a mutant to confirm the assertion, but this observation likely indicates that p600 in *Drosophila* is not required for survival into adulthood.

A 1996 study by Richards *et al.* was the first to directly assess the role of p600 (as the *pushover* mutant) in the nervous system [22]. In addition to the recurring observation that *p600* mutant males were sterile, the authors reported that the *p600* mutants did not right themselves when overturned (hence “*pushover*”), to fly when dropped, or to escape repeated prodding [22]. The authors go on to demonstrate convincingly that p600 acts presynaptically at the neuromuscular junction by showing that its homozygous mutation results in a higher frequency of spontaneous vesicle release, and larger induced excitatory postsynaptic currents and potentials [22]. Further study of p600 in the nervous system of *Drosophila* showed that p600 mutants (as *pushover*) had thickened perineurial glia (*i.e.* glia in the peripheral nervous system of *Drosophila* embryos) [23]. This thickening was synergistic with the *inebriated* and *ether-a-go-go* mutants, encoding an unknown neurotransmitter transporter and a potassium channel respectively [23]. Thus it was concluded that the increased glial growth was in response to increased neurotransmitter signalling and/or persistence.

The only information on the subcellular role of p600 in *Drosophila* can be derived from a 1998 study in which p600 (under the name calossin/ CALO, a ligature of *calmodulin* and *colossal*) was detected in a screen for calmodulin-binding proteins in a retinal expression library ([24]; details on calmodulin binding in section 1.1.9). This study also reported that p600 mRNA was expressed nearly exclusively in the adult (*cf.* similar mammalian expression in [10]). In this determination the authors incidentally show that p600 mRNA migrated at 15kb, indicating that a full-length (*ca.* 5000 a.a.) product is probably translated from it. The authors also show that p600 expression falls to some 19% of wild-type levels in the head of the *sine oculis* (“without eyes”)
mutant, indicating a greater expression of p600 in the visual system of drosophila ([24]; cf. high expression in the mammalian retina, Figure 1A in Belzil et al. [4]).

From the literature that is available, one may conclude that in Drosophila p600 is a calmodulin-binding protein which is required for male fertility, regulates neuronal excitability, and is probably not required for survival into adulthood, unlike its mammalian counterpart (detailed in section 1.1.8).

1.1.3.2 BIG (A. Thaliana)

An understanding of the role of p600 in A. thaliana requires a brief introduction to auxin transport. Auxins are small-molecule plant growth hormones of which the prime and relevant representative is indole-3-acetic acid. Auxins are conveyed from one tissue to another by serial transport: It is exported by one cell, diffused through the extracellular space, imported by an adjacent cell, diffused through the cytoplasm, and exported again (the details of this process are summarized from a review [25]). Inward diffusion from the extracellular milieu, in which the acidic auxins are protonated and uncharged, may occur passively though it is facilitated by transporters [26]. Within cells however, where auxins are deprotonated and therefore cell-impermeable, auxins must be actively exported. In order for this process to be unidirectional, efflux takes place on only one side of the cell, namely the direction of transport. The resulting phenomenon, called polar auxin transport, is thereby able to convey auxins at a rate of about 1 centimeter in an hour [27] across any tissue that expresses a functioning exporter. Since the process is so largely passive, a defect in auxin signalling is most likely attributed to faulty export.
The first mention of p600 in *A. thaliana* (as the *doc1* mutant) was in a 1994 study in which randomly generated *p600* mutants showed stunted growth [28]. A 1997 study reported an independently-generated *p600* mutant (as *tir3*), which also reported a general growth defect, and more specifically a total absence of lateral roots [29]. Since it was known that the development of lateral roots is dependent on auxin signaling, the authors conducted a series of experiments culminating in the conclusion that in *p600* mutants the polar transport of auxin was disrupted, while the sensitivity thereof was unaltered. A 2001 study supported the finding of a transport defect in *p600* (*tir3*) mutants and further showed that in these mutants the protein PIN1, which is normally asymmetrically localized in the plasma membrane as part of the polar auxin transport system, was drastically mislocalized to an intracellular compartment [30]. This was however only apparent when auxin efflux was inhibited by a small molecule with an unknown target, making a direct interpretation impossible. Recent reports have further described auxin-dependent growth defects in *p600* mutants in the inflorescence and other aerial organs [31], [32], but offered no significant mechanistic insights.

Auxin transport would, in a mammalian context, be a misnomer. It is better considered to be a “serial export”, since the actual movement of the auxin molecule is by diffusion. Thus the role of p600 in *A. thaliana* seems to be as a regulator of an export process, though it is far from clear how it acts. To this end it was demonstrated that the localization of the PIN1 auxin carrier was under control of p600 [30], and that the p600 mutant phenotype was replicated by blocking endomembrane transport with brefeldin A [33]. Brefeldin A has a variety of activities, chief among them to regulate the dynamics of the Golgi apparatus [34]. Scialy *et al.* showed that brefeldin A treatment causes a collapse of the Golgi apparatus into the endoplasmic reticulum
(ER), thereby explaining the many observations that brefeldin A inhibits transport form the ER to the Golgi apparatus [35]. Since the ER/Golgi system is responsible for the production and localization of membrane proteins, including those that export auxin, this system must function properly in order for auxin transport to occur. This idea is further supported by findings that treatment brefeldin A [34] phenocopies the absence of lateral roots seen in p600 mutants [33]. Thus the auxin transport defect in A. thaliana may be quite generalizable to many different cell types and organisms, if p600 is acting as a regulator of endomembrane transport. Any discussion of the role of p600 in A. thaliana must however be qualified by pointing out that the C-terminal portion shares only 52% sequence similarity to the mammalian protein while the N-terminal portion is, to a good approximation, unrelated [5].

### 1.1.4 The role of p600 in protein degradation and autophagy

There are two major pathways of protein degradation in the cell, the proteasomic and autophagic. The former recycles a majority of proteins, some 80-90%, and tends to handle more short-lived proteins [36]. The latter handles more long-lived proteins, and also aggregated proteins [36]. p600 is involved in both pathways.

#### 1.1.4.1 The role of p600 in the N-end rule pathway

Proteins are targeted for proteasomic degradation by being covalently bound to ubiquitin. This process is mediated by the actions of three classes of proteins: The ubiquitin-activating enzyme (E1) forms a high-energy bond with ubiquitin which it can then transfer to a class of ubiquitin-carrier proteins (E2). The ubiquitin-protein ligase (E3) then targets E2 to the condemned protein,
catalyzing the transfer of K48-linked ubiquitin to lysine residues on the condemned protein [37]. This signal is necessary and sufficient for proteasomal degradation (summarized from [15] pp.939-940). The E3 component of this pathway is therefore responsible for determining which proteins are to be degraded. The identity of the N-terminal residue often serves as the degradation signal, or degron, to which the E3 component directly binds in order to target it for ubiquitinylation ([15] p.940). A number of post-translational processes determine the final N-terminus of the protein, and thus its longevity (the specifics of this process, including N-degron identities, are reviewed in [38]). This so-called N-degron is recognized by N-recognins, the E3 components that target proteins for degradation. The general importance of the N-terminus in determining the fate of proteins is called the N-end rule pathway.

In 2005 Tasaki et al. identified p600 (as UBR4, “ubiquitin protein ligase E3 component n-recognin 4”) as an E3 N-recognin [18]. There are however important qualifications to this finding. p600 conspicuously lacks the RING domain, a variant of the Zn-finger domain found in other E3 ligases [18]. In other E3 ligases the RING domain is responsible for interacting with the E2 carrier and for the actual ligase activity [37]. It was however shown that p600 directly binds to destabilizing (arginine and phenylalanine) N-degrons, and that knockdown of p600 in cultured fibroblasts increased the longevity of proteins with destabilizing N-degrons [18]. There is therefore an apparent contradiction in that p600 fills the role of the E3 ligase, but lacks the required domain. There are two possible ways of reconciling these facts: First, by supposing that p600 has a domain similar in function to the RING domain, but which is so different in structure as to be unrecognizable by sequence alignment. Second, by supposing that p600 is part of a larger E3 complex in which it performs the N-recognin function, while another protein associates with E2 and/or assumes the actual ubiquitin-ligase function.
The 26S proteasome, a massive (2GDa) dumbbell-shaped adenosine triphosphate (ATP)ase complex, degrades ubiquitinylated proteins [39]. Besche et al. showed that p600 associates directly with the 26S proteasome complex in a tight but substoichiometric manner [40]. The BioGRID database for protein interactions lists 9 independent observations of interactions between p600 and polyubiquitin chains, demonstrating a tight link between p600 and proteins targeted for degradation ([41] for the database entry; references listed therein). Though superficially intuitive it is in fact unusual, though not unprecedented, that an E3 ligase would interact directly with the proteasome [40]. Together these results show that p600 is not only responsible for tagging proteins for proteasomal destruction, but that it exists in complex with the proteasome (summarized in figure Figure 1.3).
Figure 1.3. The role of p600 in proteasomal degradation. A) p600 is associated tightly with the 26S proteasome. As an N-recogin, it detects unstable N-degrons of proteins which it targets for degradation. B) As an E3 component, p600 is presumed to direct an E2 ubiquitin carrier to the protein which is targeted for degradation. C) Guided by p600, the E2 ubiquitin carrier and an unknown ligase catalyze the addition of ubiquitin to lysine groups on the target protein. D) The ubiquitinylated protein is then targeted for destruction by the 26S proteasome. (Summary figure based on the finding that p600 is associated with the 26S proteasome [40] and acts as an E3 N-recogin [18]. The general mechanics of ubiquitin-mediated degradation are depicted as described in reference [15] pp.939-940)
1.1.4.2 The role of p600 in autophagy

Tasaki et al. studied p600 as a regulator of autophagy in the mouse embryo [42]. Amino acids are supplied to the embryo as proteins which are imported and degraded in autophagic vesicles. At embryonic day 9.5 in the mouse embryo, this importation and degradation is handled by an endoderm-derived population of cells in the yolk sac [42]. Tasaki et al. have recently reported that p600 is enriched in this cell population in the mouse embryo, and that it colocalizes with their autophagic vesicles [42]. The authors further show that in cultured fibroblasts, p600 knockdown increases the expression of the autophagic microtubule-associated protein 1A/1B-light chain 3 (LC3), generates a large number of autophagic vesicles, and enhances the autophagic degradation of target proteins. All of these effects were enhanced when autophagy was induced by serum starvation. More work will be required to sort out how p600 regulates autophagy, and accordingly the study in question [42] drew only the conservative conclusion that p600 regulates autophagy.

1.1.5 The role of p600 in cell adhesion

Focal adhesions are large complexes that link the actin cytoskeleton with the extracellular matrix. The proteins pathways involved are numerous and complex, but largely converge on the Focal Adhesion Kinase (FAK) [43]. FAK phosphorylation at Y397, which enables it to interact with other focal adhesion proteins, is drastically decreased in cells wherein p600 is knocked down [44]. The study which reported this finding also reported a number of defects in cellular morphology which, though circumstantial, could plausibly result from defects in actin or focal adhesions [44]. It is unclear whether p600 is acting directly on FAK to alter its phosphorylation state, or upstream (e.g. by degrading a regulator of FAK phosphorylation).
1.1.6 The role of p600 in viral transformation

p600 appears in the literature in the transformation mechanisms of two viruses, the Dengue virus and the papillomavirus. Beyond their superficial similarity, their mechanisms appear to be wholly unrelated. They do however warrant study in that they appear to be extensions of the physiological roles of p600.

1.1.6.1 Dengue virus

Dengue virus RNA is recognized directly by intracellular receptors, initiating a cascade of signalling resulting in the release of interferon α/β. This signal acts on the releasing and neighbouring cells to initiate a signalling cascade culminating in the transcription of a set of genes that enact an antiviral response. During Dengue virus infection of mammalian cells there is an initial interferon-mediated response, but it is suppressed as the infection progresses. (the above was summarized from [45]). This pattern indicated that the virus suppresses the interferon response against it and indeed it was found to degrade signal transducer and activator of transcription 2 (STAT2), the dimerization of which is one of the final and necessary steps of interferon production [46]. A more recent study showed that p600 is co-opted by the Dengue virus protein NS5 in order to cause the degradation of STAT2, resulting in greater viral replication [47]. p600 could thus generally be used to destroy host cell immunity in other infections due to its role in protein degradation. The authors of the study however show that the NS5 proteins in the related yellow fever and West Nile flaviviruses are both unable to bind p600, and the replication of these viruses was predictably unaffected by p600 knockdown [47]. This study is however of general interest, beyond the area of viral transformation: The finding that the
degradation of STAT2 by p600 is dependent on the NS5 protein demonstrates that p600 has the ability to target specific substrates for degradation.

1.1.6.2 Papillomavirus
The papillomavirus genome consists of 7 early-expressed genes (E1-E7) and 2 late-expressed genes (L1 & L2) [48]. In order to replicate the viral DNA, the E7 protein binds the retinoblastoma protein [49], leading to its degradation [50]. This disrupts its repression of the transcription factor E2F-1, allowing the cell to enter S-phase [48]. p600 was identified as an interactor of the retinoblastoma protein [44], and later found to also bind the E7 protein directly [51], [52]. The purpose of this interaction is however unclear. What is certain is that p600 is required for anchorage-independent growth, a characteristic phenotype of papillomavirus transformation [51], [52]. However though the degradation of the retinoblastoma protein is mediated by the proteasome [50], knockdown of p600 does not increase the lifespan of retinoblastoma protein, nor of the E7 protein [52]. The mechanism by which p600 assists transformation by papillomaviruses is therefore open to speculation, though the requirement for p600 for anchorage independent growth is a promising lead (see section 1.1.7.1).

1.1.7 The role of p600 in cell survival
1.1.7.1 p600 prevents apoptosis due to cell detachment
Adherent cells use the contact with the extracellular matrix as a survival signal. Loss of this contact initiates a programmed cell death event called anoikis [53]. Loss of this cell-matrix requirement is a characteristic of transformation by the papillomavirus, and a hallmark of the malignant transformation caused by carcinogenic strains of the human papillomavirus [48], [50].
Since anchorage-independent growth caused by the papillomavirus proteins E6 and E7 requires p600, it follows that p600 prevents anoikis in transformed cells.

The evidence for a mechanism by which p600 prevents anoikis is mostly open to speculation. If indeed p600 has a role in cell adhesion signalling (see section 1.1.5) then it may be required to transduce the anti-apoptotic signal generated by cell adhesions. p600 may also have a far more general role in the execution of programmed death.

1.1.7.2 p600 prevents apoptosis due to serum-starvation
Nakatani et al. [44] showed that knockdown of p600 in cultured fibroblasts was sufficient to cause a small but significant population of cells to undergo apoptosis, and that this effect was greatly increased when the cells were grown in serum-free medium. Serum starvation is however too crude of an experimental manipulation, and apoptosis too broad of a target, to conclude much about the events occurring in between. This finding nevertheless illustrated that p600 can be an essential protein.

1.1.8 Characterization of the p600 knockout mouse
Three p600 knockout mice have been generated to date: The group of Y.T. Kwon [42] generated a knockout by inserting a cassette in the region encompassing exons 36-42 (as shown in their online supplemental information), and that of Y. Nakatani generated a constitutive knockout by excision of exon 1, and a conditional knockout using the same targeting cassette to insert loxP elements flanking exon 1 [54]. Our own breeding program also used the latter strategy and confirmed the knockout phenotype, elaborating particularly on brain defects (Belzil et al. [4]; see Appendix 4).
1.1.8.1 p600 is an essential protein

The group of Tasaki et al. found no p600 knockout mice beyond embryonic day (E) 11.5 [42]. Nakaya et al. found that p600 ablation was lethal beginning at E11.5, and totally lethal by E13.5 [54]. A conditional knockout was then generated in an attempt to circumvent this early lethality, which was thought to be caused by placental defects (see below). In this model p600 ablation is driven by the SRY-box 2 (Sox2) promoter, which is expressed early in development, but not in extra-embryonic tissue [55]. This paradigm delays embryonic death, though p600 ablation is still universally lethal between E11.5 and E14.5 [54]. It is therefore to be concluded that p600 is an essential protein. The successful delay of lethality by employing the Sox2/cre conditional knockout model supports the assertion of Tasaki et al. that death results from a failure of embryos to recover maternal nutrients via autophagy in the extra-embryonic tissue [42].

1.1.8.2 p600 is required for mouse embryonic development

Tasaki et al. were sparing in their description of the p600 knockout phenotype, only reporting on vascular defects: At E8.5 embryos appear normal, while by E9.5 they develop a pallor [42]. The yolk sac contains angiogenic defects which they ultimately attribute to a failure of autophagy-enriched yolk sac cells which express p600 (see section 1.1.4.2).

The report of Nakaya et al. [54] provides more detailed descriptions: The constitutive knockout was shown to be notably smaller from E10.5 onward. They specifically assessed the placental labyrinth, which is a functional successor to the endoderm-derived yolk sac layer described in Tasaki et al. [42] in that it handles maternal nutrient exchange [56]. The labyrinth was found to be much smaller and to contain vascular defects. This problem was circumvented in the Sox2/cre conditional knockout. p600^{SC-/-} embryos were still much smaller, and sporadically
developed bulges on their heads ([54]; Figure 1.4). In the heart, the interventricular septum failed to fuse with the endocardial cushions by E12.5 and E13.5, leaving a canal between the ventricles. The ventricular wall was thinned in the conditional knockout, with an approximately 40% reduced cell count. In the conditional knockout fewer proliferating cells were found, though there were no more apoptotic cells. FAK Y397 phosphorylation was markedly reduced in the heart, suggesting a role in cell adhesion [54]. The liver of the conditional knockout similarly shows reduced cell counts, and consequently has more empty space in the tissue. Finally, Nakaya et al. report that at E13.5 the brain is much smaller, mostly compressed in the rostrocaudal direction, with a net reduction in area of more than 50%. The ventricles are also shown to be much smaller. The lateral ganglionic eminences are cavitated in the conditional knockout, apparently as a result of cell death [54]. Immunohistochemistry in Belzil et al. [4] shows that the thickness of the cortical plate is greatly diminished in the p600 conditional knockout at E13.5, as evidenced by reduced staining against Tuj-1, a marker for newly-born neurons (Figure 1.5).
Figure 1.4. *p600* conditional knockout mice (left) are smaller than their wild-type counterparts (right), and sporadically present with protrusions on their heads. Scale bar = 1mm (published in Nakaya et al. [54] as Figure 3C. No permission is required for its inclusion here)
Figure 1.5. The p600 sox2/cre conditional knockout (p600\textsuperscript{SC−/−}) brain is significantly smaller, is more malformed, and has a much thinner cortical plate than that of the heterozygote (p600\textsuperscript{SC+/−}). Tuj-1 staining for newly-born neurons in E13.5 wild-type brain transverse sections shows expression in the telencephalon (TE), diencephalon (DE) and hindbrain (HB). The lateral ventricle (LV) is shown for comparison. Zoom-in of the insets (black box) illustrates a drastic thinning of the cortical plate in the telencephalon of p600\textsuperscript{SC−/−}, mice populated with fewer Tuj-1-positive neurons when compared to p600\textsuperscript{SC+/−}. Scale bars = 500 µm, 40 µm (inset). (immunohistochemistry by C.B., Figure 6 in [4]. No permission is required for its inclusion here)
1.1.9 p600 as a calmodulin-binding protein

p600 was identified in the retina of *D. melanogaster* as a binding partner of calmodulin (CaM), the archetypical Ca\(^{2+}\)-detector [24]. Based on this report, its equivalent in mammalian cells was tested for the same function, wherein it was shown that mammalian p600 binds CaM only in the presence of Ca\(^{2+}\) [44]. These reports demonstrate two important points. Firstly, that in spite of the meagre conservation of p600 between mammals and *D. melanogaster*, CaM binding is preserved. This indicates that CaM-binding is fundamental to p600 function, and further hints that the phenotype of the *D. melanogaster* p600 mutants may be generalizable to mammals. Secondly, these findings show that p600 is in principle able to detect a rise in intracellular Ca\(^{2+}\) by binding Ca\(^{2+}\)/CaM. This adds a contingency to the hypothetical role of p600, meaning that it may have the ability to change its function in response to changes in the concentration of intracellular Ca\(^{2+}\).

1.1.10 The role of p600 in neurons and in the brain

The 2008 paper by Shim *et al.* [10], the first to assess the role of p600 in the mammalian (mouse) brain, is in many ways the direct ancestor to the work contained in this thesis. Its principal conclusions, which form the basis for the work in Chapters 2 and 3, are outlined in sections 1.9.1-1.9.4.

1.1.10.1 p600 is enriched in the brain and expressed in neurons

In 2005 Tasaki *et al.* compared the expression of full-length p600 in various tissues, demonstrating that its expression is by far greatest in the brain and testes [18]. To this finding, Shim *et al.* [10] added two important trends in the expression of p600. First, that its expression
increases over the course of development, reaching its peak in the adult brain and spinal cord. Second, that within the brain it is expressed in neurons. These results were the first to suggest that p600 may play a role in adult neurons in mammals. Shim et al. furthermore showed that p600 was expressed in various areas of the brain, including the hippocampus.

1.1.10.2 p600 is a microtubule-associated protein

Shim et al. were the first to report that p600 is a microtubule (MT)-associated protein [10]. This was demonstrated in several ways: First, that cloned C-terminal fragments of p600 are co-purified with MT fractions when expressed in CAD cells. Second, that these same C-terminal fragments, when expressed in and purified from E. Coli, are capable of polymerizing purified MTs. Thirdly, that immunogold immunocytochemistry followed by electron microscopy shows full-length p600 adhering to MTs in primary cortical neurons. Though three cloned fragments of p600 show MT-binding activity, two of these overlap significantly and may share the same MT-binding region. The conclusion must therefore be that there are at least two binding regions, one between residues 3214 and 3899, another between residues 3910 and 5183 (Figure 1.2). There may of course be several MT-binding domains within these regions.

Perhaps equally significant is the finding by Shim et al. that depletion of p600 in neurons leads to decreased tubulin acetylation. Tubulin acetylation is a marker of MT stability, and can in fact be caused by the stabilization of MTs with paclitaxel [57] or with the expression of MT-stabilizing proteins [58]. The finding that p600 knockdown decreases MT acetylation is therefore significant in that it demonstrates that p600 regulates the stability of the MT cytoskeleton in neurons.
1.1.10.3 p600 associates with the endoplasmic reticulum

Similar techniques were used to establish that p600 associated with the endoplasmic reticulum (ER). Again, subcellular fractionation was used to establish that a fraction of p600 cofractionates with the ER. Electron microscopy also showed full-length p600 associating with membranous tubular structures. Transfection of cloned fragments of p600 showed that a fragment representing residues 1681-2401 shows strong affinity for the ER by immunocytochemistry and subcellular fractionation, and that a fragment representing residues 3214-3899 shows partial affinity (Figure 1.2).

The findings that p600 interacts both with MTs and the ER are by no means contradictory, since the ER has a close and dynamic relationship with MTs, the former being constantly drawn out and held under tension by the latter [59]. The exogenous expression of different fragments of p600 has the advantage of isolating the competing affinities that exist within the full-length protein. Based on these two results, it seems probable that p600 is, or is present at, the junction between MTs and the ER (see discussion section 4.2.1).

1.1.10.4 p600 is required for neurite outgrowth and neuronal migration

If p600 is indeed responsible for the stability of MTs, and if it has a role in transporting or distributing the ER, processes dependent on these functions would be expected to fail when p600 is knocked down. The first area of investigation in Shim et al. was the extension of neurites. The base of the neurite growth cone is enriched in ER membrane [60], which is transported in tubules along MTs [59]. Consistent with a requirement for p600 in MT stability and/or ER transport, Shim et al. showed that neurite extension is blocked when p600 is depleted by RNA interference (RNAi). The second area of investigation was in migrating neurons, wherein the leading process
acts analogously to an extending neurite. In neurons depleted of p600, the leading process was found to be deficient of ER and visibly distorted. The migration of p600-depleted newly-born neurons was disrupted, with significantly fewer migrating to the middle and upper cortical plate and more stalled in the intermediate zone. p600 is therefore required for neurite outgrowth and migration in newly-born neurons. Shim et al. [10] concluded that it was likely, though not certain, that the failures in neurite extension and neuronal migration in p600-depleted neurons resulted from a failure in MT stability or ER transport.

1.1.1 Summary of the p600 protein and hypothesis
The composite picture of p600 is of a large protein that is associated with MTs and the ER. It has a variety of subcellular roles, though no obviously unifying theme. We do however know that p600 is enriched in the brain [18] and expressed in neurons [10]. Since neurons express a large complement of Ca\(^{2+}\) channels, and p600 is in principle able to detect a rise in intracellular Ca\(^{2+}\) by virtue of its binding to CaM, the actions of p600 in neurons may be Ca\(^{2+}\)-dependent. Finally, since p600 is required for the survival of the mouse embryo [42], [54] and of cultured cells [44], I have hypothesized that p600 promotes survival in neurons through its interaction with Ca\(^{2+}\)/CaM. Since they have been shown to express p600 [10], and contain well-characterized Ca\(^{2+}\) signalling pathways, this hypothesis has been tested in hippocampal pyramidal neurons. The following sections therefore discuss the Ca\(^{2+}\) handling properties of these neurons.

1.2 Ca\(^{2+}\) signalling and neuronal survival in hippocampal neurons
A rise in the concentration of intracellular Ca\(^{2+}\) induced by glutamate is sufficient to cause neuronal degeneration, a phenomenon known as excitotoxicity [61]. A failure of neuronal Ca\(^{2+}\)-
handling systems leads to cell death through several pathways (reviewed in [62], [63]). While there are thousands of proteins involved in the dynamics of Ca\(^{2+}\) buffering, binding and signalling that result in neuronal death, the start and end points of the process are schematically simple: Ca\(^{2+}\) ions enter the cytosol from the extracellular space or from intracellular reservoirs, and cause death either by programmed death or by necrosis. These two origins and two outcomes of elevated intracellular Ca\(^{2+}\) are discussed in the following subsections, in order to provide context for the hypothetical role of p600 as a Ca\(^{2+}\)-dependent protein which responds to excitotoxic Ca\(^{2+}\) entry and prevents neuronal death.

1.2.1 The origins of excitotoxic Ca\(^{2+}\)

The diffusion of the Ca\(^{2+}\) ion in the cytosol is severely limited in both the total distance it can travel as well as the time it travels before it is bound and sequestered [64]. Therefore Ca\(^{2+}\) sources and their detectors must be located in close proximity. This is true for the initiation of Ca\(^{2+}\)-induced cell death cascades: Ca\(^{2+}\) build-up incurred by influx through N-methyl-D-aspartate (NMDA) receptors is universally fatal in neuronal culture, while an equivalent Ca\(^{2+}\) load through voltage-dependent channels is drastically less so [65]. This importance of the origin of an elevation of the cytosolic Ca\(^{2+}\) concentration means that if p600 promotes survival in response to Ca\(^{2+}\) influx, it must be responsive to Ca\(^{2+}\) influx through channels which are known to cause excitotoxicity in hippocampal neurons. The following two sections therefore discuss the extracellular and intracellular sources of potentially excitotoxic Ca\(^{2+}\) elevation.
1.2.1.1 Extracellular $\text{Ca}^{2+}$ entry

$\text{Ca}^{2+}$ may enter the neuronal cytosol through ligand or voltage-gated channels. In the context of this study the former category includes three kinds of glutamate receptors, the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), kainate, and NMDA receptors. The AMPA receptor is found in high abundance in the postsynaptic density [66], mediating the majority of fast excitatory transmission. The kainate receptor is found postsynaptically in only certain cell types (pertaining to this study, only in CA3 pyramidal neurons [67], [68]). While kainate and AMPA receptors are typically rendered impermeable to $\text{Ca}^{2+}$-conductance in hippocampal neurons by RNA editing, NMDA receptors conduct a substantial and sustained $\text{Ca}^{2+}$ current ([69], pp.214-215). NMDA receptors additionally control the opening of pannexin hemichannels which conduct a large nonspecific cation current [70]. Given the substantial contribution of NMDA receptors to excitotoxicity in hippocampal neurons, if p600 acts as a $\text{Ca}^{2+}$-dependent promoter of neuronal survival it would be expected to be responsive to $\text{Ca}^{2+}$ influx through these receptors.

Of the substantial variety of voltage-dependent $\text{Ca}^{2+}$ channels (VDCCs), only the L-type and the T-type are broadly expressed in the soma and dendrites of hippocampal neurons [71] and thus able to contribute to the general rise in intracellular $\text{Ca}^{2+}$ with which this study is concerned. The T-type channel conducts a small $\text{Ca}^{2+}$ current that is completely deactivated during sustained depolarization [72]. There is some limited evidence that T-type blockade protects neurons from degeneration in both ambient hippocampal culture conditions [73] and oxygen/glucose deprivation of hippocampal slices [74]. Because they conduct a small current and rapidly inactivate during a sustained depolarization, T-type channels are unlikely to substantially contribute to a rise in $\text{Ca}^{2+}$ sufficient to cause neuronal degeneration, especially in the
experimental paradigms used in these studies. L-type channels however conduct a much larger and more sustained $\text{Ca}^{2+}$ current, and are thus at face value a better candidate for effecting a neurodegenerative cascade. The evidence in this regard is however conflicting. Some studies have reported a neuroprotective effect of L-type VDCC blockade after ischemic injury [75], which has been associated with excessive neuronal $\text{Ca}^{2+}$ influx. L-type VDCC blockade also protects against passive degeneration in neuronal cultures [76]. There are however indications that the L-type VDCC is a poor candidate for the initiation of $\text{Ca}^{2+}$-dependent neuronal death. $\text{Ca}^{2+}$-bound CaM limits the L-type current, creating a negative feedback loop [77]. The presence of this feedback loop suggests that L-type VDCCs are by design restricted from contributing to pathological amounts of $\text{Ca}^{2+}$ entry. Direct activation of VDCCs also fails to initiate acute degeneration comparable to agonists of the NMDA receptor [65]. L-type $\text{Ca}^{2+}$ influx is thus to be treated as a plausible but minor origin for the initiation of a neurodegenerative cascade.

1.2.1.2 $\text{Ca}^{2+}$ release from intracellular stores

The principal intracellular calcium store, the ER, contains a free $\text{Ca}^{2+}$ concentration of 0.2-1 mM [78]. Additionally, several proteins such as calreticulin and calsequestrin contain up to 50 low-affinity ($K_d$ ca. 1 mM) $\text{Ca}^{2+}$ binding sites, creating an additional pool of $\text{Ca}^{2+}$ able to equilibrate with the cytosol [79]. Egress principally occurs through three paths: Through one of two well-characterized $\text{Ca}^{2+}$-conducting channels, or through a poorly-defined leak. As mentioned above, $\text{Ca}^{2+}$ is sequestered in the endoplasmic reticulum by the action of the sarco-/endoplasmic reticulum $\text{Ca}^{2+}$ ATPase. Inhibition of this pump causes the ER to equilibrate with the cytosol, probably by way of several leak channels (reviewed in [80]). The second exit for luminal $\text{Ca}^{2+}$ is through the ryanodine receptor (RyR), whose opening is induced by an elevation in $\text{Ca}^{2+}$. This
so-called “calcium-induced Ca\(^{2+}\) release” has been show to significantly contribute to the rise in intracellular Ca\(^{2+}\) concentration both during the action potential, and more locally during synaptic stimulation (evidence summarized in Table 2 of [79]). The third method of egress is by activation of the inositol 1,4,5-triphosphate IP3 receptor (IP3R). The signalling cascade, as it pertains to this thesis, begins with the binding of glutamate to metabotropic glutamate receptors (mGluRs) 1 and 5, G-protein coupled receptors which are expressed post-synaptically [81] and in the hippocampus [82]. This binding releases G\(_q\), thereby activating phospholipase C, which in turn cleaves the phospholipid phosphoinositol 4,5-bisphosphate to generate IP3 ([83] and [27], p. 860). The IP3R is, like the RyR, sensitive to the concentration of Ca\(^{2+}\). The relationship is complex, however, and depends on the concentration of both Ca\(^{2+}\) and IP3 [84].

Because of the complex properties of ER Ca\(^{2+}\) management, the role of ER Ca\(^{2+}\) release in excitotoxicity is difficult to predict. Depletion of luminal Ca\(^{2+}\) stores through the leak pathway is sufficient to cause apoptosis in some neuronal cell lines (e.g. [85]) but not in others. In some cases this is due to a rise in cytosolic Ca\(^{2+}\) but in others it is apparently due instead to the decline in luminal Ca\(^{2+}\) [86]. In some systems, depletion of luminal Ca\(^{2+}\) can even prevent neuronal death by precluding channel-mediated release [87]. It is not clear whether ryanodine and IP3 receptors even share the same pool of Ca\(^{2+}\) [79], and if they are therefore able to deliver the same amount of Ca\(^{2+}\) to the cytosol. They are certainly not localized similarly: On an anatomical level, the IP3R is more prevalent in the CA1 of the hippocampus, whereas the RyR is enriched in the CA3 [88]. The IP3R is localized to dendritic spines [89], and is therefore an excellent candidate for mediating an excitotoxic post-synaptic rise in cytosolic Ca\(^{2+}\). The RyR seems rather to be localized more presynaptically [90], and accordingly seems to regulate excitotoxicity by controlling glutamate release [91]. RyRs can however also elevate Ca\(^{2+}\) postsynaptically [79]. In
cortical cultures and organotypic slices, NMDA-induced excitotoxicity is mitigated by antagonists against both ryanodine and IP3 receptors, at least at lower concentrations of NMDA [92]. Ca\(^{2+}\) efflux from the ER is therefore to be considered an entirely plausible source of excitotoxic Ca\(^{2+}\) influx to the cytosol of hippocampal neurons, and p600 may therefore hypothetically show a Ca\(^{2+}\)-dependent response to it.

1.2.2 Mechanisms of Ca\(^{2+}\)-induced neuronal death

The mechanism of Ca\(^{2+}\)-induced death in neurons is related to the severity of the excitotoxicity, and probably lies along a continuum of necrosis and programmed cell death [93]–[95]. If p600 is promoting survival in neurons, one way it may act is by opposing Ca\(^{2+}\)-induced death pathways. Conversely, p600 loss of function would be expected to activate neuronal death pathways. Therefore in order to understand the role of p600 in Ca\(^{2+}\)-induced survival, descriptions of necrotic and programmed cell death appear below, with the understanding that they are by no means exclusive states.

1.2.2.1 Necrotic neuronal death

Necrotic death entails the following characteristics (summarized from [96], [97] and [98]): Chromatin is not cleaved as it is in programmed cell death, indicating that endonucleases are not activated. Cytoplasmic vacuoles form and mitochondria swell. The plasma membrane is permeabilized, allowing cellular components to leak into the extracellular space. Descriptions of necrosis focus on two principle events in necrosis [97]: The first is the loss of ionic homeostasis, a necessary consequence of mitochondrial failure, as sustaining sufficient ionic gradients requires oxidative phosphorylation. The second is the activation of normally-latent proteases.
The canonical examples are calpains, specific Ca\textsuperscript{2+}-dependent endoproteases [99], and the far less specific cathepsins [100], which are released from lysosomes during necrosis [97]. It is not known at what point a necrotic cell can be pronounced dead, though either the loss of ionic homeostasis or the activation of broadly-acting proteases is probably sufficient to destroy the cell.

1.2.2.2 Programmed neuronal death

Canonical apoptosis begins with the release of cytochrome c from the inner mitochondrial membrane, facing the outer mitochondrial membrane [101]. Two steps are required for its release into the cytosol: The neutralization of its cardiolipin membrane anchor, and the permeabilization of the outer mitochondrial membrane [101]. This permeabilization of the outer mitochondrial membrane requires Bax and/or Bak, two nuclear-encoded proteins that are able to form channels in the outer mitochondrial membrane, and perhaps even conduct cytochrome c directly [102]. Once in the cytosol cytochrome c along with Apaf-1 and procaspase-9 forms a 7-spoked wheel-like structure called the apoptosome [103]. Upon nucleotide-binding, procaspase-9 is activated to become caspase-9, which in turn cleaves and activates the executioner caspase, caspase-3 [103]. These activated caspases have many targets, including a variety of cytoskeletal targets and the endogenous inhibitor of the endonuclease responsible for the cleavage of chromatin [104], [105]. In addition to this canonical mechanism, there is evidence for caspase-independent apoptosis following excitotoxicity: The nuclear enzyme PARP-1 mediates the mitochondrial release of AIF, initiating a caspase-independent apoptosis in response to NMDA exposure [106], [107]. This type of apoptosis apparently relies on a controlled release of AIF from mitochondria analogous to cytochrome c release, as evidenced by the anti-apoptotic effect
of the Bax/Bak repressor Bcl-2 [107]. As with necrosis, it is not entirely clear what event kills
the cell: Chromatin cleavage is probably sufficient, but not necessary for death [108]. The
actions of the apoptotic proteases may be sufficient either by targeting specific proteins or by the
sum of their activity, but this is also unclear.

In addition to these apoptotic mechanisms, other programmed cell death mechanisms
appear to exist. For example, it would appear as though Ca\(^{2+}\)-activated calpain actively
suppresses caspase activation [109]. This active repression of another death program suggests
coordination, though the extent of the replacement programme is unknown. Endoplasmic
reticulum stress caused by a number of stressors such as elevated Ca\(^{2+}\) influx, oxidative stress
and protein aggregation (reviewed in [110]), can also initiate apoptosis by acting on
mitochondria [111]. Kainate excitotoxicity has been shown to initiate this ER stress pathway in
hippocampal neurons, and stress inhibition is protective against apoptosis [112]. The causes of
this program are unknown, and it appears to commit cells to die before cytochrome c is released,
so it may act through a pathway distinct from canonical apoptosis [111]. There is also evidence
that autophagic flux is blocked in hippocampal excitotoxicity, and that enhancing it is
neuroprotective [113]. Apparently contrary evidence shows that rapid NMDA-triggered
hippocampal excitotoxicity shows large-scale activation of autophagy [114]. Such observations
spur the ongoing debate about the role of autophagy in neurodegeneration: While autophagic
vacuoles are prominent in many degenerative paradigms and human disorders, their presence
may be an attempt by neurons to mitigate damage and avoid death (see review, [115]). More
evidence will be required to understand the role of autophagy in excitotoxic neuronal death.
However since p600 is a known regulator of autophagy [42], loss of p600 could possibly lead to
the activation of neuronal death with autophagy.
1.2.3 The interplay between the microtubule cytoskeleton, Ca\textsuperscript{2+} and cell death

MTs constantly cycle between slow growth and rapid collapse, a phenomenon known as dynamic instability. Though this is a property of the polymer itself and will thus occur \textit{in vitro}, MTs are much more dynamic in cells due to the actions of MT-associated proteins ([27] pp. 929-936), such as p600 [10]. An understanding of how Ca\textsuperscript{2+} signalling pathways interact with the microtubule cytoskeleton therefore offers means to understand the role of p600 as a Ca\textsuperscript{2+} signalling protein.

1.2.3.1 Ca\textsuperscript{2+} levels affect microtubule stability

An elevation of cytosolic Ca\textsuperscript{2+} can alter the stability of MTs in several ways. Firstly, purified MTs cannot polymerize in the presence of millimolar Ca\textsuperscript{2+} [116]. While on purified MTs Ca\textsuperscript{2+} promotes MT collapse at 0.5-0.6 mM, an inhibitory effect is evident on impure (MT-associated protein-containing) MTs at lower concentrations of 0.1 mM [117]. These concentrations vastly exceed the global physiological range of Ca\textsuperscript{2+}, though it is entirely possible that local concentrations of Ca\textsuperscript{2+} reach titres sufficient to directly act on MTs. Ca\textsuperscript{2+} can also destabilize MTs at much lower concentrations. For example, during ischemia the Ca\textsuperscript{2+}-dependent protease calpain is activated, and cleaves the dendritic MT-stabilizing protein MAP2 [118], [119]. The phosphorylation of the archetypical low-molecular weight MT-binding protein tau promotes the disassembly of MTs [120]. The control of tau phosphorylation is at least partially under the control of Ca\textsuperscript{2+}-dependent kinases. Immediately following neuronal depolarization, tau is transiently phosphorylated, followed by a more sustained dephosphorylation [121]. Tau is directly phosphorylated by the Ca\textsuperscript{2+}/CaM-dependent kinase II (CaMKII) in its MT-binding domain [122], significantly reducing its ability to bind MTs [123]. CaMKII itself directly
phosphorylates tubulin, favouring its depolymerization [124], [125]. Thus over a broad range of Ca$^{2+}$ concentrations, several Ca$^{2+}$-activated components are capable of effecting changes on MT stability.

1.2.3.2 Microtubule stability affects Ca$^{2+}$ levels and neuronal survival

The reverse scenario is also true, that the stability of MTs can affect Ca$^{2+}$ influx: Pre-treatment with the MT-stabilizing compound paclitaxel mitigates the glutamate-induced rise in intracellular Ca$^{2+}$ in hippocampal neurons, apparently by restricting Ca$^{2+}$ influx through AMPA receptors [126]. The MT-destabilizing compound colchicine is, as would be expected, neurotoxic [127]. Its toxicity can be significantly, but only partially, mitigated by preventing extracellular Ca$^{2+}$ influx [126]. This data shows that MTs are potentially important as a regulator of excitotoxicity, which carries the implication that MT-associated proteins such as p600 may thereby regulate excitotoxicity.

1.2.4 The pathological Ca$^{2+}$-dependent aggregation of CaMKII$\alpha$

CaMKII is a Ca$^{2+}$-signalling protein which is highly enriched in hippocampal neurons [128]–[132]. Each polypeptide chain of CaMKII consists of an N-terminal catalytic domain, a C-terminal association domain, and an autoregulatory domain between the two [133]. The binding of Ca$^{2+}$/CaM to the autoregulatory domain is sufficient to activate CaMKII [133]. Of the different CaMKII genes, CaMKII$\beta$ is predominantly found in the brain, and CaMKII$\alpha$ is found almost exclusively in the brain [134]. Together, CaMKII$\alpha$ and $\beta$ account for more than 90% of the Ca$^{2+}$/CaM-dependent kinase activity in the rat brain [135] and are essentially the only CaMKII genes expressed therein [133]. As a ubiquitous Ca$^{2+}$ signalling protein and another
CaM-interacting protein, the p600 may act as a Ca\(^{2+}\)-signalling protein via CaMKII. Furthermore, several studies have reported that when exposed to pathological conditions such as those that would precede neuronal death, CaMKII\(\alpha\) loses its catalytic activity and instead enters an aggregated state. The entry into this aggregated state may therefore serve as a proxy for excitotoxicity. The evidence for the entry of CaMKII\(\alpha\) into this aggregated state, and its utility as a pathological marker, is discussed below.

1.2.4.1 Aggregation of purified CaMKII\(\alpha\)

Hudmon et al. demonstrated aggregation of CaMKII purified from rat forebrain [136]. In this study, the aggregation was accomplished in acidic conditions (pH 6.5) and low ATP (10 µM), while an elevation in either was sufficient to prevent aggregation. As a favourable point of comparison with other systems, the aggregation, autophosphorylation, and loss of activity took place within 5-7 minutes (30°C) of the beginning of the autophosphorylation reaction. Nevertheless, the data of Hudmon et al. [136] shows that purified CaMKII is intrinsically sensitive to pH and to the concentration of ATP.

A follow-up study by the same group, which used light-scattering to monitor the aggregation of CaMKII real-time, added several findings [137]. First, they demonstrated a concentration-dependence of CaMKII aggregation. They demonstrated aggregation in low concentrations of <0.5 µM, rising exponentially thereafter (cf. 50-100µM CaMKII in neurons) [137]. Secondly, they demonstrated that CaMKII\(\beta\) has essentially no propensity to aggregate under these conditions. Thirdly, they show that in the absence of ATP CaMKII inactivates of its own accord, but only aggregates once a small amount of ATP is added. This shows that although the two effects occur in tandem, they are distinct. Thirdly, they show that CaM is required for
aggregation. Lastly, they show that the expression of the CaMKII autoregulatory domain, prevents aggregation while the expression of a peptide substrate does not. Together, these two studies [137], [138] show that CaMKIIα aggregates when CaMKII is bound by Ca²⁺/CaM in conditions of low pH, when its autoinhibition is lifted, and that this is a behaviour of the purified enzyme.

1.2.4.2 Aggregation of CaMKIIα in cell culture

The direct successor to the in vitro studies used a model wherein GFP-CaMKIIα was transfected into human embryonic kidney (HEK) cells [139]. Here aggregation was induced by fixing intracellular pH at 6.5 and intracellular [Ca²⁺] at 2 mM. The time course of aggregation in HEK cells compares favourably with the aggregation of the purified enzyme, with aggregation beginning in the first few minutes and reaching completion by the 5th minute (cf. [137] and [139]). During glutamate treatment of neurons, particularly NMDA receptor stimulation, intracellular Ca²⁺ rises and intracellular pH drops [140], [141]. Meeting both of the conditions required for CaMKII aggregation, glutamate-treatment would be predicted to induce CaMKII aggregation. The authors of the study show that this is indeed the case in cultured rat neurons [139]. They furthermore show, using several CaMKII mutants, that the autophosphorylation mimic T286D does not aggregate, while T286A, which cannot be phosphorylated, has a higher propensity to aggregate. This makes aggregation mutually exclusive with the previously-established mechanism of autophosphorylation (T286) of the enzyme, wherein it moves to synapses and remains catalytically active [142]. This fact is confirmed by the demonstrably non-synaptic character of the aggregation when induced by glutamate stimulation [139].
Another group discovered non-synaptic CaMKII aggregation in the soma of hippocampal neurons by using the mitochondrial uncoupler (proton ionophore) CCCP [143]. They found that these aggregates could be purified to homogeneity. In a follow-up study, this group showed that mitochondrial uncoupling caused an elevation in intracellular \(\text{Ca}^{2+}\), and that this was responsible for the aggregation of CaMKII [144]. Together, the data from cell culture systems illustrates a remarkable similarity between the behaviour of the purified enzyme, and that expressed in living cells.

1.2.4.3 The aggregation of CaMKII\(\alpha\) \textit{in vivo}

The conditions for the aggregation of CaMKII are met \textit{in vivo} during ischemia: During ischemia, there is a rapid drop in pH and rise in intracellular \([\text{Ca}^{2+}]\) [145], and a depletion of ATP [146]. Early work showed a rapid precipitation of CaMKII from ischemic brain homogenates, in conjunction with its inactivation [147], [148]. The time course for this precipitation was similar to that seen using the purified enzyme and to that seen in cell culture [137], [139], reaching a maximum by 5 minutes [147]. The aggregation was remarkably persistent: CaMKII is depleted from the soluble fraction of the hippocampus for some 2 hours after a brief 10 minute ischemic episode [147]. The \textit{in vivo} data is therefore consistent with the behaviour of CaMKII in cell culture and of the purified enzyme. Along with the aggregation of CaMKII, these data show a pronounced and persistent inactivation.

1.2.4.4 A putative molecular mechanism for the aggregation of CaMKII\(\alpha\)

It cannot be directly proven that the aggregates seen with the purified enzyme, in cultured neurons, or those spun out of ischemic brains represent the same kind of CaMKII aggregation.
There is however good cause to believe that they are the same since they all form in a remarkably similar time-scale, and since aggregation is an autonomous behaviour of the enzyme itself that will occur when the conditions of low pH, high Ca\(^{2+}\) and low ATP are met.

The mechanics of aggregation are unknown, though there is good evidence for a mechanism. The binding of CaM is required for CaMKII aggregation, as evidenced by the failure of the purified protein to aggregate without it [137], and the failure of the T305/306D mutant to aggregate in HEK cells [139]. The aggregates are persistent and do not dissipate of their own accord, but can be undone by the chelation of Ca\(^{2+}\) [139]. Both the catalytic (N-terminal) and association (C-terminal) domain are required for aggregation [139], suggesting bonding between those two domains. The pH-dependence of the phenomenon implies that there is a protonation event, and the range of 6.5-7.5 implies the involvement of a histidine residue. H282 is a fine candidate: It is located in the autoregulatory region, and its protonation presumably (based on H282R mutants) frees the catalytic domain [149]. Freeing of the catalytic domain is at most a necessary condition, but not a sufficient one since the phospho-mimic mutant T286D is incapable of aggregation [139]. Though the aggregates appear to be autophosphorylated at T286, which ought to prevent aggregation, this is probably explained by the fact that only some subunits of the holoenzyme are phosphorylated [139], [150] (by analogy, CaMKII\(\beta\) is found in the aggregates [143] in spite of the fact that it cannot aggregate itself). The requirement for the involvement of the catalytic domain is supported by the finding that competition from the autoregulatory domain, which also inhibits the catalytic domain, precludes aggregation [137]. Perhaps the most telling evidence of the conformation of the enzyme during aggregation is that the T286A mutant, which has no Ca\(^{2+}\)/CaM-independent activity, has a higher propensity to aggregate than the wild-type enzyme [139]. The working model is therefore that upon exposure
to elevated intracellular Ca$^{2+}$, there is an opposing action between autophosphorylation at T286, which confers upon it catalytic activity and synaptic localization (reviewed in [133]), and the sequestration in inactive and non-synaptic aggregates, depending on the pH and the concentration of ATP.

1.2.4.5 CaMKIIα aggregation as a pathological indicator

Entry into an aggregated state can reasonably be supposed to preclude other physiological behaviours of CaMKIIα. Even allowing for the possibility that CaMKIIα in its aggregated form is able to interact with its usual binding partners, we can assume that these interactions would be greatly diminished on account of its inability to diffuse freely. Directly to this point, several studies show that this aggregation is accompanied by catalytic inactivation [138], [147]. Since CaMKIIα aggregation is caused by the low pH and high Ca$^{2+}$ seen in excitotoxicity, since it persists so long as intracellular Ca$^{2+}$ is elevated, and since it is an autonomous behaviour of the enzyme, aggregation may be used as an indicator of entry neuronal into an excitotoxic state. As a hypothetical regulator of Ca$^{2+}$-dependent neuronal survival, the loss of p600 function ought therefore to first cause CaMKIIα aggregation prior to actual neuronal death.

1.2.5 Summary

The hypothesis that p600 promotes survival in hippocampal neurons by its interaction with CaM has been explored in the two manuscripts which comprise the following two chapters of this thesis. The first chapter addresses the role of p600 in neuronal survival, and its interactions as a Ca$^{2+}$ signalling protein. This study also uses CaMKIIα aggregation as a marker in order to study the mechanism by which p600 prevents neurons from entering into a pathological state.
The second study further explores this phenomenon using single cell depolarization and live imaging, and investigates the role of the MT-stabilizing function of p600.
Chapter Two: A Ca\(^{2+}\)-dependent mechanism of neuronal survival mediated by the microtubule-associated protein p600

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2.1 Summary

In acute and chronic neurodegeneration, Ca\(^{2+}\) mishandling and disruption of the cytoskeleton compromise neuronal integrity, yet abnormalities in the signaling roles of cytoskeletal proteins remain largely unexplored. We now report that the microtubule-associated protein p600 (also known as UBR4) promotes neuronal survival. Following depletion of p600, glutamate-induced Ca\(^{2+}\) influx through NMDA receptors, but not AMPA receptors, initiates a degenerative process characterized by endoplasmic reticulum fragmentation and endoplasmic reticulum Ca\(^{2+}\) release via inositol 1,4,5-trisphosphate receptors. Downstream of NMDA receptors, p600 associates with the calmodulin/calmodulin-dependent protein kinase II\(\alpha\) complex. A direct and atypical
p600/calmodulin interaction is required for neuronal survival. Thus, p600 counteracts specific 
Ca\textsuperscript{2+}-induced death pathways through regulation of Ca\textsuperscript{2+} homeostasis and signaling.

2.2 Introduction
Neuronal survival requires controlled Ca\textsuperscript{2+} signaling/homeostasis and the maintenance of 
cytoskeletal integrity. In acute (traumatic brain injury, stroke) and chronic neuronal degeneration 
(Alzheimer disease, Parkinson disease), perturbations in Ca\textsuperscript{2+} handling trigger neuronal death 
through common routes, including excitotoxicity and oxidative and endoplasmic reticulum (ER) 
stress (1–7). Genetic and biochemical alterations in cytoskeletal proteins are also observed in a 
wide diversity of chronic neurodegenerative disorders (Alzheimer disease, Parkinson disease, 
etc.) featuring deregulation of Ca\textsuperscript{2+} homeostasis and signaling (1, 5, 8). For instance, mutations 
at highly conserved residues within or near the microtubule-binding domain in the human 
microtubule-associated protein Tau gene cause inherited frontotemporal dementia and 
parkinsonism (9). Furthermore, the hyperphosphorylation of Tau by Ca\textsuperscript{2+}-activated kinases 
reduces its ability to stabilize microtubules and its aggregation in toxic oligomers and/or fibrils 
(10). Although abnormalities in the neuronal cytoskeleton and Ca\textsuperscript{2+} mishandling both contribute 
to acute and chronic neuronal death, alterations in the signaling functions of cytoskeletal proteins 
remain largely unexplored.

Upon exposure to glutamate, the influx of Ca\textsuperscript{2+} through NMDA and AMPA receptors, 
and subsequently via VDCCs, activates secondary messenger systems to propagate the 
Ca\textsuperscript{2+}signaling cascade, leading to cytoskeletal changes, plasticity, and survival. However, 
excessive influx of Ca\textsuperscript{2+} via these receptors and channels triggers neuronal death via cytoskeletal 
disruption, whereas drugs that antagonize them prevent cell death (2, 5, 7, 11). Inside the neuron,
the ER constitutes the primary intracellular source for Ca\(^{2+}\) mobilization and signaling. ER Ca\(^{2+}\) levels are controlled by Ca\(^{2+}\) release through ryanodine and inositol 1,4,5-triphosphate (IP3) receptors and the activity of inward pumps (5). These collectively determine the ER functioning mode and, consequently, neuronal survival. As the ER is transported on microtubules (12), collapse of microtubules is linked to ER fragmentation and dysfunction (13, 14).

We have recently discovered a novel microtubule-associated protein in CNS neurons: Protein 600 (p600, also known as UBR4) (15). p600 is required for neurite outgrowth and neuronal migration in the developing neocortex (15), two events that concomitantly require cytoskeletal dynamics and Ca\(^{2+}\) signaling. Thus, we hypothesized that p600 protects against Ca\(^{2+}\)-induced neuronal dyshomeostasis and degeneration by maintaining structural stability and Ca\(^{2+}\) homoeostasis.

2.3 Materials and methods

2.3.1 Culture of dissociated primary hippocampal neurons

All animals were housed and handled according to Canadian Council on Animal Care guidelines and experimentation approved by the Health Sciences Animal Care Committee. Hippocampi were dissected from perinatal rat pups of either sex. Tissues were dissociated and plated on 24-well plates in basal medium Eagle (Invitrogen) supplemented with penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 10% FBS (Invitrogen), and 10 mM HEPES (pH 7.3) to a final density of 200,000 cells/well. Cultures were incubated until DIV10 with two partial medium changes.
2.3.2 Generation and characterization of RNAi and mCherry-ER vectors

RNAi sequences were selected based on the criteria of Ambion, Inc. Complementary hairpin sequences were commercially synthesized and cloned into pSilencer 2.0 under the U6 promoter (Ambion). Sequences for the rat p600 RNAi are base pairs AAGCAGTACGAGCCATTCTAC and AAGCCTGTCAAGTACGATGAA. A random sequence without homology to any known mRNA was used for control RNAi. All RNAi constructs were tested in primary neuronal cultures by both Western blot and immunofluorescent staining. In order to achieve the higher transfection efficiency required for Western blotting (Figure 2.1C), the Amaxa nucleofector system was used as described previously (16). The mCherry vector was a gift from Dr. R. Y. Tsien. The mCherry cDNA was subcloned into pcDNA 3.1 flanked by an N-terminal calreticulin ER-targeting sequence and a C-terminal ER retention KDEL tag as described previously (17). ER-specific expression was confirmed by BiP co-immunofluorescence in transfected HeLa cells.

2.3.3 Transfection, glutamate treatment, and assessment of GFP-CaMKIIα aggregation

Hippocampal cultures were transfected with vectors encoding one of two RNAi sequences directed against p600 or with a control RNAi along with rat GFP-CaMKIIα in a 4:1 ratio. 16 h post-transfection, cultures were treated with bath solution (135 mM NaCl, 5 mM KCl, 2 mM MgCl2, 3 mM CaCl2, 10 mM D-glucose, 10 mM HEPES, pH 7.3) with or without 30 µM Glu, 3 µM Gly for 3 min, washed for 2 min with PBS, fixed, and scored with a fluorescent microscope. Aggregation was quantified as a percentage of neurons showing any form of non-synaptic CaMKIIα aggregation relative to total GFP-positive neurons. All neurons were scored with a Nikon T2000E fluorescent microscope using a x40 or x60 objective such that there was no ambiguity as to whether the transfected cells were neurons or glia (confirmed by NeuN staining;
Figure 2.1D). For better comparison between culture sets, data on GFP-CaMKIIα aggregation are mostly presented as normalized values.

2.3.4 Quantification of neuronal degeneration

In order to assess neuronal survival, cultures were transfected as described above, except GFP-CaMKIIα was replaced with pEGFP N2. After the prescribed incubation time, cultures were fixed in 4% paraformaldehyde in PBS, and GFP-positive neurons were counted as a measure of neuronal survival. In the experiment reported in Figure 2.1E, wherein neurons were depleted of p600 by RNAi, neurons were transfected for 16 h and counted 6 h thereafter. The assessment of survival with p600P peptide (Figure 2.5B) required a longer incubation of 24 h due to the higher expression requirement for competitive inhibition by the peptide.

For analysis of necrosis, neurons were transfected for 16 h, incubated in growth medium substituted with the vital dye propidium iodide (1 µg ml⁻¹; Molecular Probes) for 15 min, washed twice with PBS, and then fixed. Propidium iodide-positive cells were all rounded but still GFP-positive. For analysis of apoptosis, neurons were transfected for 16 h and then returned to growth medium for 6 h, at which point caspase-3 activity is maximal in similar culture systems (18). Neurons were then fixed and immunostained with an antibody directed against cleaved caspase-3 (9661, Cell Signaling).

2.3.5 Quantification of ER fragmentation

In order to quantify the relationship between CaMKIIα aggregation and ER fragmentation, cells were co-transfected with GFP-CaMKIIα and an ER-targeted fluorophore, mCherry-ER, and treated as described above. The measure of bimodal bias was used to quantify CaMKIIα aggregation and ER fragmentation. For calculation of the bimodal bias, the pixel density outputs
of the GFP-CaMKIIα fluorophore were analyzed using NOCOM (19) to assess bimodality of pixel distribution, a measure of aggregation. The difference between the likelihood of a single-mode fit versus that of a bimodal fit of the pixel density, the so-called bimodal bias, was used as a measure of pixel/protein aggregation.

2.3.6 Pharmacological treatments

Dissociated primary hippocampal neuronal cultures (DIV10) were transfected with p600 RNAi and GFP-CaMKIIα in a 4:1 ratio. 16 h post-transfection, cultures were incubated with a bath solution containing a compound of interest or a vehicle control for 15 min (except EGTA, for which a 2-min preincubation was used). Cultures were then treated with a bath solution containing the compound of interest/vehicle control and 30 µM Glu, 3 µM Gly for 3 min. Cultures were then washed for 2 min with PBS and fixed, and GFP-CaMKIIα aggregation was scored as described above. All compounds were from Sigma except nimodipine and ryanodine (Enzo).

2.3.7 p600 blocking peptide

Primary hippocampal neuronal cultures (DIV10) were co-transfected with GFP-CaMKIIα vector and an empty vector (pcDNA3.1+), a pcDNA3.1+ vector encoding the p600/calmodulin (CaM) interaction sequence (residues 4086–4113 on human p600 (MAPSKSELRLYLTEKYVWRWKQFLSRRG)), or the same vector containing an additional negative control, the blocking peptide with a W4103E mutation on a conserved residue on the CaM-binding motif (MAPSKSELRLYLTEKYVERWKQFLSRRG) in a 1:2 ratio. 16 h post-transfection, cultures were treated with bath solution with or without 30 µM Glu, 3 µM Gly for 3
min, washed for 2 min with PBS, fixed, and scored for prevalence of CaMKIIα aggregation with a fluorescent microscope. In order to assess the effect of the p600/CaM blocking peptide on survival, cultures were transfected as above, with pEGFP N2 substituted for GFP-CaMKIIα. 16 h post-transfection, they were treated with bath solution with or without 30 µM Glu, 3 µM Gly for 3 min and returned to growth medium for 24 h. Where indicated, this growth medium was supplemented with 100 µM 2-APV (Sigma) or 20 µM benzylxycarbonyl-VAD-fluoromethyl ketone (Millipore). Neuronal survival counts are normalized within each experiment and expressed as a percentage in order to compare values between experiments.

2.3.8 Statistical analyses
The correlation between CaMKIIα aggregation and ER fragmentation was analyzed with two-tailed Pearson’s correlation. Data sets acquired by comparison of neuronal cultures were checked for normality by the Anderson-Darling test. Data sets that were not normally distributed were compared by the Mann-Whitney U test. Data generated by scoring CaMKIIα aggregation were found by Bartlett’s test to have unequal variance between test groups and hence were compared by two-tailed Welch’s t test. All other normally distributed data were compared by two-tailed Student’s t test. Curve fitting was done in MATLAB (MathWorks).

2.3.9 Western blots and in situ hybridization
The mice were sacrificed by intraperitoneal injection of chloral hydrate. Protein extracts of the animals or primary hippocampal neurons were obtained by homogenization in SDS-urea (0.5% SDS, 8 M urea in phosphate buffer, pH 7.4) or radioimmunoprecipitation assay lysis buffer with a mixture of protease inhibitors (PMSF, leupeptin, pepstatin, aprotinin) and 1 mM sodium
orthovanadate for phosphatase inhibition. The protein concentration in tissue and cell homogenates was estimated by the Bradford procedure (Bio-Rad). Proteins (20–50 μg) were fractionated on 6, 8, or 10% SDS-PAGE and blotted on a nitrocellulose or PVDF membrane for Western blot analysis. Membranes were incubated with antibodies against p600 (F4, F7) (15), actin (C4, MAB1501, Chemicon), synaptophysin (MAB329, Chemicon), CaMKIIα (MAB8699, Chemicon), PSD-95 (75-028, NeuroMab), and calmodulin (05-173, Millipore). Quantifications were corrected with levels of actin and performed with the Labscan program (Image Master 2D software version 3.10, Amersham Biosciences). The in situ hybridization was performed as described previously (20) with both sense and antisense probes.

2.3.10 Subcellular fractionation

Mouse brain was homogenized in 5 ml of 10 mM HEPES, 0.32 M sucrose (with the addition of 0.2 mM PMSF, 1 mM EGTA, and a complete protease inhibitor tablet (Roche Applied Science)) at 1000 x g at 4 °C for 10 min. Separated from the pellet (P1), the supernatant (S1) was then centrifuged again for 15 min at 12,000 x g. The resulting pellet (P2) corresponds to the crude synaptosome preparation, whereas the supernatant (S2) corresponds to the cleared soluble cytosolic fraction.

2.3.11 Gel filtration assay

Recombinant Xenopus laevis calmodulin was prepared as described previously (21). A peptide derived from p600 encompassing the sequence identified by the CTDB search program (SKSELRLHLYLTEKYVWRWKQFLSRRG) was synthesized by the Queen’s University Peptide Synthesis Laboratory (Kingston, Canada). For non-denaturing urea PAGE analysis, calmodulin
and p600 peptide at several molar ratios were incubated for 1 h at room temperature in 1 mM Tris, pH 7.5, 50 mM CaCl2, and 10 M urea. The latter was included to disrupt nonspecific interactions. Electrophoresis was performed as described previously (22).

2.3.12 Immunoprecipitations
For the experiments testing the effect of glutamate pretreatment on the interaction between p600 and CaMKIIα, the medium was replaced with KRH buffer (containing 128 mM NaCl, 5 mM KCl, 1 mM NaH2PO4, 2.7 mM CaCl2, 1.2 mM MgSO4, 10 mM glucose, and 20 mM HEPES (pH 7.4)) for 30 min at 37 °C. Neurons were then treated with 100 µM/10 µM glycine in Mg2+-free KRH buffer for 5 min and then were lysed in radioimmune precipitation assay buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing protease and phosphatase inhibitors. The homogenized cells were centrifuged at 13,000 x g for 5 min at 4 °C. The resulting supernatant was collected for co-immunoprecipitation. 1 mg of protein was used per immunoprecipitation. Immunoprecipitations were performed as described (16) and according to standard protocols.

2.4 Results
2.4.1 p600 is enriched in the brain and required for neuronal survival
In mice, the protein levels of p600 are highest in the brain (Figure 2.1A). In the adult cortex and hippocampus, p600 is predominantly expressed in neurons (15) (Figure 2.1B). The embryonic lethality of two different p600 null mouse strains (24, 25), however, precluded an investigation of the mechanistic roles of p600 in adult brain neurons but hints at the requirement of p600 for cell survival in vivo. Because of this lethal phenotype and expression of p600 in the adult
hippocampus (15) (Figure 2.1B), we sought to use primary cultured hippocampal neurons to study the roles of p600 in neuronal survival. In our experimental settings, DIV10 neurons had differentiated, elaborated synapses and responded to glutamate treatment, indicating functional maturity (see “Materials and Methods”).

We first sought to establish a loss-of-function methodology by which we could study the role of p600 in a culture system. First, we established the efficacy of an RNAi knockdown strategy. In primary hippocampal neurons electroporated with a vector encoding one of the two p600-specific small interference RNA oligonucleotides directed against p600 mRNA (15) (RNAi 1 or 2), endogenous p600 protein was diminished by 60–70%, as revealed by Western blots (Figure 2.1 C) (15). The strong decrease in p600 was further confirmed at the single cell level by immunofluorescent staining followed by confocal microscopy (15) (data not shown). No decrease in p600 was observed in cells treated with a random oligonucleotide sequence without homology to any known mRNA (control RNAi).

In order to test the hypothesis that p600 is required for neuronal survival, we transfected DIV10 primary hippocampal neurons with a control or a p600 RNAi vector together with pEGFP N2 and scored cultures for number of viable neurons 22 h after transfection. Under normal culture conditions, primary mature hippocampal neurons depleted of p600 degenerate more than control RNAi-treated neurons (where control RNAi = 100 ± 13% survival, p600 RNAi 1 = 58.1 ± 7.5% survival, t_{10} = 2.87, p = 0.017; Figure 2.1E). This result was confirmed with p600 RNAi 2 (where control RNAi = 100 ± 10% survival, p600 RNAi 2 = 65.9 ± 10% survival, t_{10} = 2.39, p = 0.038).

We next explored the manner of neuronal demise. By microscopy, we found that depletion of p600 by RNAi did not increase the number of cells permeable to propidium iodide.
(where control RNAi = 100 ± 24%, p600 RNAi = 96.5 ± 13%, t_{10} = 0.41, p = 0.69) or increase the number of cells showing cleaved caspase-3 immunoreactivity (where control RNAi = 100 ± 31%, p600 RNAi = 74.6 ± 51%, t_{10} = 0.14, p = 0.89; Figure 2.1E). We thus conclude that neurons depleted of p600 undergo a nonapoptotic and non-necrotic demise (see “Discussion”).
Figure 2.1. p600 is enriched in the brain and required for neuronal survival. A, p600 is enriched in the brain, as detected by Western blots. B, in situ hybridization of coronal mouse adult brain sections shows the mRNA expression pattern of p600. Pyr, pyramidal layer of the hippocampus; Dg, dentate gyrus; Cx, cortex; Pir, piriform cortex; Tha, thalamus; Pvn, paraventricular nucleus; Sch, suprachiasmatic nucleus; Son, suprachiasmatic nucleus; Dm, dorsomedial nucleus; Ag, amygdala; Hyp, hypothalamus; Hb, habenula. The sense probe did not produce any signal, providing specificity to the observed pattern of expression. C, two short hairpin RNA (shRNA) constructs targeting mouse p600 mRNA (15) tested by electroporation in primary hippocampal neurons effectively knock down p600 expression. Note the absence of difference in levels of CaMKIIα, activated CaMKIIα (phospho-Thr286-CaMKIIα; p-CaMKIIα), and the microtubule-associated proteins Lis1 and Ndel1 between untreated control RNAi and untreated p600 RNAi-treated neurons, indicating specific loss of p600. Actin was used as a control for protein loading. Values are shown ± S.D. (error bars); **, p (T< t) two-tailed 0.01. D, at the magnification used for scoring neuronal survival and GFP-CaMKIIα aggregation, neurons (top) were readily distinguished from glia (bottom). Morphological differences in transfected cells (green) were confirmed by the presence or absence (arrowheads) of NeuN staining (red). Scale bar, 10 µm. E, When depleted of p600 by RNAi, 42% of neurons die over the time frame indicated in the experimental timeline below. There were no significant differences in the number of neurons with cleaved caspase-3, an indicator of apoptosis, or which were permeable to propidium iodide (P.I.), an indicator of necrosis (see values and statistics under “Results”). Shown are means ± S.E. (error bars) of six repeats. ~200 neurons were analyzed per condition.
2.4.2 Glutamate-treated hippocampal neurons exhibit Ca\(^{2+}\)-induced dyshomeostasis when depleted of p600

Having found that p600 is required for neuronal survival, we determined to look at the events preceding this demise. Because the survival of hippocampal neurons is so highly susceptible to perturbations in Ca\(^{2+}\) homeostasis, we hypothesized that p600 was regulating Ca\(^{2+}\) homeostasis in these cells. For this purpose, we used GFP-CaMKII\(\alpha\) as a marker for neuronal dyshomeostasis. In whole ischemic brains, CaMKII\(\alpha\) forms high molecular weight aggregates (26). CaMKII\(\alpha\) aggregation can also be induced within minutes by toxic insults, specifically by increasing intracellular [Ca\(^{2+}\)], energy depletion (of ATP) or by lowering intracellular pH (26 – 32). This somatic/dendritic aggregation is distinct from the synaptic clustering of CaMKII during heterosynaptic plasticity, as evidenced by a decrease in CaMKII\(\alpha\) activity (33). Under the microscope, these non-synaptic aggregates appear as puncta distributed throughout the cell soma and processes and are therefore easily detected in fixed or live cells. The aggregation of GFP-CaMKII\(\alpha\) is therefore a convenient biomarker for neuronal dyshomeostasis and an early correlate of neuronal demise.

In order to induce a rise in intracellular calcium by physiologically relevant means, we used glutamate treatment. We first constructed a dose-response curve in which we determined that neurons depleted of p600 by RNAi are more susceptible to undergoing GFP-CaMKII aggregation (Figure 2.2A). Based on this finding, we selected a dose of 30 µM Glu, 3 µM Gly (3 min), to which control RNAi-transfected neurons were generally unresponsive by the metric of GFP-CaMKII\(\alpha\) aggregation. Neurons depleted of p600 by RNAi are, however, ~2.5-fold more likely to exhibit CaMKII\(\alpha\) aggregation than neurons transfected with control RNAi following treatment with 30 µM Glu, 3 µM Gly (control RNAi versus p600 RNAi 1: 100 ± 24 and 239 ±
65%, \( t_8 = 5.04, p = 0.0010 \); control RNAi versus p600 RNAi 2: 100 ± 16 and 330 ± 67%, \( t_{10} = 3.34, p = 0.020 \); Figure 2.2, B and C). Two p600 RNAs targeting different sequences of p600 mRNA generated the same result, thereby providing specificity to the observed phenotype.

Based on the role of p600 in microtubule stabilization (15), we first tested whether stabilization of microtubules with 100 nM paclitaxel could rescue p600 depletion-induced GFP-CaMKII\( \alpha \) aggregation. Intriguingly, paclitaxel was found to have no effect (Table 2.1). This result indicates that upon glutamate treatment, p600 does not maintain neuronal homeostasis by stabilizing microtubules. Based on our previous finding that p600 associates with the ER (15), we hypothesized that p600 maintains ER structure in response to Ca\(^{2+}\) influx. To test this, we co-transfected neurons depleted of p600 by RNAi with GFP-CaMKII\( \alpha \) and an mCherry-tagged ER (mCherry-ER) construct. This allowed us to simultaneously visualize the aggregation of the kinase and the fragmentation of the ER reported in degenerating neurons (4, 5, 34, 35). Using a quantification of bimodal bias (see “Materials and Methods”) to quantify kinase aggregation and ER fragmentation, we found a strong positive correlation between these two pathocellular processes in glutamate-treated p600-depleted neurons (Figure 2.2, D and E); cells with high levels of kinase aggregation also exhibit a high degree of ER fragmentation. This constitutes one of the early Ca\(^{2+}\)-induced events observed in p600-depleted neurons and presages their demise.

Analysis of GFP-CaMKII\( \alpha \) aggregation also provided a means of rapidly assessing the manner of involvement of Ca\(^{2+}\) entry while avoiding the long term effects of pharmacological treatments. To this end, primary hippocampal neurons transfected with control or p600 RNAi 1 were treated with the Ca\(^{2+}\) chelator EGTA or a vehicle prior to treatment with Glu/Gly for 3 min and scored for CaMKII\( \alpha \) aggregation after a 2-min rinse. Chelation of extracellular Ca\(^{2+}\) with EGTA significantly rescued CaMKII\( \alpha \) aggregation in these cells (Table 2.1). These results
revealed that entry of extracellular Ca\(^{2+}\) renders p600-depleted cells vulnerable to Ca\(^{2+}\)-induced dyshomeostasis.

Glu/Gly treatment triggers Ca\(^{2+}\) influx through NMDA receptors and Ca\(^{2+}\)-permeable AMPA receptors. To determine which of these receptors are involved in the Ca\(^{2+}\)-induced dyshomeostasis phenotype, we treated p600-depleted cells with the NMDAR inhibitors 2-APV and MK-801 or the AMPA/kainate inhibitor CNQX prior to 3-min Glu/Gly treatment and then assessed CaMKII\(\alpha\) aggregation after a 2-min rinse. We found that 100 µM 2-APV rescued kinase aggregation by 80%, whereas 1 µM MK-801 almost completely abolished the formation of the aggregates (Table 2.1). Blockade of L-type VDCCs with 50 nM nimodipine also had a minimal yet significant effect in blocking the formation of the aggregates. In contrast, blockade of the AMPA and kainate receptors with 10 µM CNQX had no effect (Table 2.1). These results revealed that NMDA receptors are the primary source of Ca\(^{2+}\) influx in p600-depleted neurons.

The fragmentation of the ER in glutamate-treated and p600-depleted cells (Figure 2.2, D and E) suggests that Ca\(^{2+}\) release from the ER contributes to the observed response. ER Ca\(^{2+}\) levels are controlled by Ca\(^{2+}\) release channels, such as ryanodine receptors and IP3 receptors, that have been shown to protect against excitotoxicity when blocked (4). No difference in CaMKII\(\alpha\) aggregation was observed between p600-depleted neurons treated with vehicles or the ryanodine receptor inhibitors dantrolene or ryanodine (10 and 50 µM, respectively; Table 2.1). However, inhibition of IP3 receptor with 50 µM 2-APV reduces the number of neurons with GFP-CaMKII\(\alpha\) aggregation by over 50%. These results indicate that IP3 receptors, but not ryanodine receptors, contribute to neuronal CaMKII\(\alpha\) aggregation caused by p600 depletion. Finally, to test whether CaM-dependent activation of CaMKII\(\alpha\) is critical for p600 RNAi-induced dyshomeostasis, we treated the neurons with 1 µM KN-62 or 1 µM KN-93, two CaMKII
inhibitors that prevent the binding of CaM to CaMKIIα. Interestingly, these drugs had no effect on CaMKIIα aggregation (Table 2.1). These results show that dyshomeostasis caused by p600 depletion does not involve CaM-dependent activation of CaMKIIα. In sum, these experiments using GFP-CaMKIIα aggregation as an early marker for Ca²⁺-induced dyshomeostasis highlighted the involvement of Ca²⁺, NMDA receptors, and IP3 receptors in a pathway initiated by the loss of p600 (Table 2.1).
Figure 2.2. Neurons depleted of p600 by RNAi undergo a rapid dyshomeostasis characterized by the aggregation of CaMKIIα and fragmentation of the ER. A, in control neurons, treatment with glutamate (supplemented with glycine, Glu/Gly, 10:1) for 3 min generates a sigmoid dose-response curve with an EC50 of 50–60 µM. Depletion of p600 by
RNAi shifts this dose-response curve leftward, indicating greater response for the same dose of glutamate. An average of 500 neurons were scored in total for each condition (control RNAi, n = 18; p600 RNAi, n = 9). B, neurons transfected with either one of the two shRNA constructs targeting p600 were found to be more susceptible to Glu / Gly-induced dyshomeostasis, as indicated by the 2–3-fold increase in CaMKII aggregation. n = 5 for control / p600 RNAi 1; n = 6 for control/p600 RNAi 2; more than 500 neurons were analyzed per condition. C, representative confocal pictures of control RNAi-treated and p600 RNAi-treated primary hippocampal neurons (DIV10) transfected with GFP-CaMKII following treatment with 30 µM Glu, 3 µM Gly for 3 min. Bar, 20 µm. D, representative confocal pictures of ER morphology visualized with an ER-targeted mCherry protein in control RNAi/GFP-CaMKII/ mCherry-ER and p600 RNAi/GFP-CaMKIIa/mCherry-ER transfected neurons following treatment with 50 µM Glu, 5 µM Gly for 3 min. The RNAi/GFP-CaMKII/ mCherry-ER ratio for transfection is 4:1:1. Bar, 10 µm. E, within single neurons, the degree of CaMKIIa aggregation correlates strongly with the degree of fragmentation of the ER (r(17) = 0.89, p < 0.0001). b.b, bimodal bias (see “Materials and Methods”). Error bars, S.E.
Table 2.1. GFP-CaMKIIα aggregation is mediated by a rise in intracellular Ca\(^{2+}\), primarily through NMDAR-mediated Ca\(^{2+}\) entry and via metabotropic ER Ca\(^{2+}\) release. Compounds that were effective in preventing GFP-CaMKIIα aggregation are shown in red. *, p < 0.05; **, p < 0.01; ***, p < 0.001; N.S., not significant (p > 0.05).
2.4.3 p600 and CaM-activated CaMKIIα associate in a Ca\(^{2+}\)-dependent manner

Degeneration in the absence of p600 requires Ca\(^{2+}\) influx through NMDA receptors, which signal through CaM and CaMKIIα. We have also previously published in vitro data showing that p600 has a Ca\(^{2+}\)-dependent association with the Ca\(^{2+}\) sensor CaM (36), which in turn has been shown to associate with CaMKIIα in a Ca\(^{2+}\)-dependent manner. We thus hypothesized that p600 physiologically associates with CaM and CaMKIIα in the presence of intracellular Ca\(^{2+}\). First, the distribution of p600, CaM, and CaMKIIα was assessed in mouse brains and primary mature hippocampal neurons. In a subcellular fractionation of adult brain, p600 predominantly co-purified with cytosolic pools of CaM and CaMKIIα (S2) (Figure 2.3A). The synaptic marker PSD-95 is used as a control, enriched in the crude synaptosomal fraction (P2) but not present in the cytosolic fraction. By immunofluorescent confocal microscopy, p600 co-localized with CaM and activated phosphothreonine 286-CaMKIIα in cell bodies and neuronal processes of primary hippocampal neurons (Figure 2.3B). These first two panels confirm that p600, CaM, and CaMKII co-localize in neurons, a prerequisite for a direct and physiologically relevant interaction. Next, we found that p600 co-immunoprecipitated with CaM and CaMKIIα from adult brain lysates (Figure 2.3C). The interactions between p600 and CaM and between p600 and CaMKII in immunoprecipitates from brain tissues were further strengthened by the exogenous application of 1 mM CaCl\(_2\) (data not shown). These results suggested that the increase in intracellular [Ca\(^{2+}\)] triggered by neuronal activity may enhance the interaction between p600 and activated CaMKIIα. To test this hypothesis, hippocampal neurons were treated with 100 µM Glu, 10 µM Gly, and co-immunoprecipitations using p600 antibodies were performed on soluble lysates derived from these cells. As shown in Figure 2.3D, a 2-fold increase in association between p600 and CaMKIIα was detected in glutamate-challenged neurons, whereas total levels
of p600 and CaMKIIα in the soluble fraction remained unaffected (-Glu/Gly: 7.9% ± 0.4%; Glu/Gly: 16.9% ± 2.2%; n = 6; **, p < 0.01 by two-tailed Student’s t test). Taken together, these results indicate that the association between p600 and Ca\textsuperscript{2+}/CaM-activated CaMKIIα is regulated physiologically by neuronal activity.
Figure 2.3. p600, CaMKII, and CaM form a glutamate-potentiated complex in hippocampal neurons. A, p600 co-fractionates with CaM and CaMKIIα in significant amounts in the cytosolic fraction (S2) but to a far lesser extent in the synaptic compartment (P2) of adult brain. Input, unprocessed homogenate. The molecular masses (kDa) of the ladder are indicated on the left. B, confocal images depicting the co-localization of p600 and CaM and phospho-Thr286 active CaMKIIα in neuronal processes and the cell body of primary rat hippocampal neurons (DIV10). Bar, 20 µm. C, CaM and CaMKIIα co-
immunoprecipitate with p600 in the adult mouse brain. CaM and CaMKIIα antibodies co-immunoprecipitate their cognate ligands (CaM, CaMKIIα, and the Cdk5 co-activator p35, which has been shown to associate with CaMKIIα (23)). Conditions with beads only (No Ab) or with species-isotopic GST antibodies were used as negative controls. D, primary hippocampal neurons at a density of 10 million/10-cm dish were treated with 100 µM Glu, 10 µM Gly, and co-immunoprecipitations using p600 antibodies were performed. A 2-fold increase in association between p600 and CaMKIIα was detected in neurons pretreated with Glu/Gly, whereas total levels of p600 and CaMKIIα remained unaffected. **, p (T < t) two-tailed < 0.01. Ab, antibody; IP, immunoprecipitation. Error bars, S.E.
2.4.4 \textit{p600 Interacts Directly with CaM}

Previously, we found that p600 interacts directly with CaM protein in a Ca$^{2+}$-dependent manner (36). These data are consistent with the report of isolation of CALO/pushover, a Drosophila counterpart of p600, in a screen for CaM-binding proteins (37). Having established a physiological Ca$^{2+}$-dependent interaction between p600 and CaM, we hypothesized that disruption of the p600/CaM interaction would lead to neuronal dyshomeostasis and death. First, to map the CaM-interacting domain of p600, we generated various p600 fragments and found that the fragment containing residues 4293–4534 interacts with CaM (data not shown). In light of the CaM binding sequence prediction algorithm available at the CaM Target Database (38), various fragments covering the C-terminal region of p600 (residues 3029–4073, 3362–3761, 3754–4158, 4048–4367, 4152–4548, 4545–4548, 4545–4868, and 4861–5183) were expressed as FLAG-tagged proteins in SF9 cells using a baculovirus expression system. In far-Western blotting, p600 fragments containing residues 3754–4158 and 4048–4367 directly bound to CaM. Two constructs expressing either the region of overlap defined by these experiments (residues 4058–4158) or a subset of this region (residues 4076–4112) were then expressed as glutathione S-transferase (GST) fusions in Escherichia coli. Far-Western blotting showed that both of the p600 fusion proteins interact with CaM, whereas GST alone does not (Figure 2.4A).

Within the p600 segment of 4076–4112, the CaM Target Database (38) identified a strong hit at residues 4089–4111 with moderate to high scores. This region does not encode any of the canonical CaM binding motifs that were well characterized previously (39) but contains conserved hydrophobic residues, such as Trp-4103. To test whether the segment comprising residues 4089–4111 constitutes a functional p600 CaM-binding site, Trp-4103 was mutated to glutamate in GST constructs containing residues 4058–4158 and 4076–4112. Far-Western
blotting showed that the mutation of W4103E in both constructs almost completely abolished the CaM binding activity (Figure 2.4A), demonstrating that Trp-4103 is a key hydrophobic anchoring residue for CaM binding. To further understand the nature of the interaction between p600 and CaM, we generated the peptide SKSELRLHLTEKYVWRWKQFLSRRG (p600P) that encompasses residues 4089–4111 of p600. Using the native gel shift titration assay method, we found that the complex was saturated at an atypical stoichiometric ratio of 1:2 CaM/p600P and that an intermediate 1:1 complex was also observed (Figure 2.4B). These data suggest that CaM could recruit two p600 molecules by virtue of two target binding sites in CaM. Taken together, our in vitro results revealed a direct interaction between p600 and CaM through residues 4088–4111 on p600 and indicate an atypical p600/CaM interaction.

2.4.5 A Direct p600/CaM interaction promotes neuronal survival

Finally, we tested whether the interaction between p600 and CaM is required for neuronal survival. In order to verify the expression of p600P, the peptide was transfected into CAD cells, a CNS-derived cell line that expresses neuronal markers (40) and shows robust expression of CaM and p600. Most importantly, these cells have sufficient transfection efficiency to assess the inhibition of the p600/CaM interaction in a lysate. They therefore served as a useful proxy for sparsely transfected hippocampal neurons. Immunoprecipitation of endogenous p600 co-precipitated CaM in the absence of Ca\(^{2+}\) (lysis buffer supplemented with 1 mM EDTA). The addition of 2 mM Ca\(^{2+}\) enhanced the amount of immunoprecipitated CaM by 66% (Figure 2.4D; \(n = 3, t_4 = 4.52, p = 0.011\) by two-tailed Student’s t test), in keeping with our previous results (Figure 2.3). By contrast, in the presence of p600P, there was a marked 78% reduction in the amount of immunoprecipitated CaM (Figure 2.4D; \(n = 3, t_4 = 13.9, p = 0.0002\) by two-tailed
Student’s t test). This finding confirmed p600P expression and validated its use as an inhibitor of the p600/CaM interaction.
Figure 2.4. p600 directly binds to CaM with atypical stoichiometry via a C-terminal binding motif. A, overlay assays for mapping of the CaM-binding domain on p600. Biotinylated CaM bound directly to GST-tagged p600 fragments (GST-p600f) containing CaM consensus: BBBØ ------ Ø ------ BØ
p600 sequence: 4084 GKAPSKSCLRHLYTEKYYWRWKQFLSR 4111
residues 4058–4158 and 4076–4112 but not to GST alone. To test if residues 4089–4111 define the p600 CaM-binding motif, the tryptophan residue at 4103 was mutated to glutamic acid in GST constructs containing residues 4058–4158 and 4076–4112 (W4103E). Overlay assays showed that the W4103E mutation in both constructs completely abolishes the CaM binding activity. The ER and microtubule-binding fragments of p600 are depicted for reference. The consensus sequence and conserved residues of the CaM-binding motif are illustrated on the atypical CaM binding motif found in p600 (B). The native gel shift titration assay method was used to define the stoichiometric ratio between CaM and p600. The p600 peptide SKSELRLHYLTEKYVWRWKQFLSRRG (p600P (residues 4088–4113)) was generated for the gel shift titration assay. The CaM-p600P complex was saturated at an atypical stoichiometric ratio of 1:2. An additional intermediate complex formed at a ratio of 1:1 was also detected. C, residues 4086–4113 of p600 (APSKSELRLHYLTEKYVWRWKQFLSRRG), containing the CaM-binding consensus sequence, p600P, is localized to the C terminus of p600. Regions containing previously defined functions are shown. D, p600P was transfected into CAD cells in order to confirm expression. Immunoprecipitation from CAD extracts using p600 antibodies effectively co-immunoprecipitates CaM, more so when 2mM Ca^{2+} (Ca^{2+} +; lane 2) is included in the lysis buffer as opposed to 1mM EDTA (Ca^{2+} -; lane 1). In the presence of the p600P motif and Ca^{2+}, the interaction between endogenous p600 and CaM is reduced by 78% (lane 4). WB, Western blot; IP, immunoprecipitation. Error bars, S.E.
We then co-transfected DIV10 neurons with p600P, an empty vector, or the mutant p600 peptide W4103E (p600P W4103E, which does not bind CaM) and GFP-CaMKIIα. Upon treatment with exogenous glutamate for 3 min (30 μM Glu, 3 μM Gly), neurons in which the p600/CaM interaction is disrupted with p600P were 35 and 54% more likely to display GFP-CaMKIIα aggregation (Figure 2.5A) when compared with the empty vector and p600P W4103E controls, respectively (U = 27, p = 0.016 and U = 17, p = 0.0015 by two-tailed Mann-Whitney U test). Predictably, transfection with the mutant p600P W4103E had no effect when compared with the empty vector (U = 48, p = 0.17). In accordance with the Ca\(^{2+}\) dependence of the p600/CaM interaction, in the absence of exogenous glutamate, there were no significant differences in the number of neurons in which CaMKIIα is aggregated (Figure 2.5A; for all comparisons, p = 0.37). 12 coverslips totaling an average of 593 cells were analyzed for each condition. From these results, we conclude that disruption of the p600/CaM interaction increases the susceptibility of neurons to undergo a Ca\(^{2+}\)-induced dyshomeostasis response symptomized by aggregation of GFP-CaMKIIα.

In order to determine if the p600/CaM interaction is required for cell survival, we transfected DIV10 neurons with p600P, an empty vector, or p600P W4103E and an enhanced GFP construct; treated with 30 μM Glu, 3 μM Gly for 3 min; and scored for cell death 24 h after treatment. The expression of p600P, but not p600P W4103E, reduced cell survival by 43%, as indicated by the significantly lower number of remaining GFP-positive cells (p600P versus empty vector control: t\(_{14}\) = 3.39, p = 0.0044; p600P versus p600P W4103E: t\(_{14}\) = 2.88, p = 0.012; Figure 2.5B). The same experiment was repeated using a sham 0 μM Glu/Gly wash but yielded a similar 44% decrease in p600P-transfected neuronal survival (p600P versus empty vector control: t\(_{14}\) = 2.28, p = 0.039; p600P versus p600P W4103E: t\(_{14}\) = 2.21, p = 0.048; Figure 2.5B).
This suggested that over the course of 24 h, the cumulative dose of endogenous glutamate is vastly in excess of the dose of exogenous glutamate, rendering it insignificant. Indeed, regardless of transfection group, our mild glutamate treatment did not create a significant difference in neuronal survival within transfection groups after 24 h (p = 0.15 for all comparisons). This finding was confirmed by repeating the 0 µM Glu/Gly treatment for 3 min and then supplementing culture medium with 100 µM 2-APV, previously shown to block GFP-CaMKIIα aggregation caused by p600 loss of function (Table 2.1). Predictably, in the presence of 2-APV, the differences in percentage of survival between cultures transfected with p600P, p600P W4103E, and an empty vector control were no longer statistically significant (p600P versus empty vector control: t_{14} = 0.627, p = 0.54; p600P versus p600P W4103E: t_{14} = 1.27, p = 0.23; Figure 2.5B).

Based on the association of p600 with the ER and its role in maintaining ER integrity, we then speculated that p600 may maintain Ca\(^{2+}\) homeostasis and neuronal survival by preventing ER stress. However, we found that in neurons in which the p600/CaM interaction is blocked with p600P, 24-h treatment with the chemical chaperone and ER stress inhibitor 4-phenylbutyrate (4-PBA) does not improve neuronal survival rates (experimental protocol as in Figure 2.5, where p600P = 100 ± 26%, p600P + 4-PBA = 87.5 ± 13%; t_{10} = 0.39, p = 0.72). We then questioned whether the glutamate-dependent aggregation of GFP-CaMKIIα upon depletion of p600 by RNAi was a result of activation of ER stress pathways. However, 4-PBA was again ineffective at preventing the aggregation of GFP-CaMKIIα (experimental protocol as in Table 2.1, where p600 RNAi + 0.002% DMSO = 100 ± 10%, p600 RNAi + 1 mM 4-PBA = 101 ± 17%; t_{8} = 0.03, p = 0.97).
Finally, although we did not find evidence of apoptosis following depletion of p600 by RNAi, we tested the possibility that over the course of 24 h, neurons were dying of delayed apoptosis. We therefore tried to rescue neuronal death in neurons transfected with p600/CaM peptide with a 24-h treatment with the pancaspase inhibitor benzyloxy carbonyl-VAD-fluoromethyl ketone (20 µM). This inhibitor, however, had no significant effect (experimental protocol as in Figure 2.5, where 0.05% DMSO vehicle = 100 ± 17%, benzyloxy carbonyl-VAD-fluoromethyl ketone = 80.7 ± 8.5%; t_{10} = 0.88, p = 0.40), confirming our earlier finding that neurons wherein p600 function is disrupted do not die by apoptotic pathways. In summary, these results indicate that physiological and non-pathological levels of glutamate-induced Ca^{2+} influx cause a direct interaction between p600 and CaM that is important to maintain neuronal homeostasis and to promote neuronal survival.
Figure 2.5. Disruption of the p600/CaM interaction causes aggregation of GFP-CaMKII followed by neuronal degeneration. A, ~30% increase in the number of neurons with GFP-CaMKIIα aggregation following transfection of the peptide and treatment with 30 µM Glu, 3 µM Gly for 3 min (the molar ratio of GFP-CaMKIIα/peptide was 1:2.4). Three experiments were performed for each condition. *, p < 0.05; **, p < 0.01; N.S., p > 0.05 (see values and statistics under “Results”). Representative confocal pictures show the effect of the p600P on CaMKIIα distribution. B, when exposed to exogenous glutamate, neurons transfected with p600P show a ~40% decrease in survival relative to empty vector-transfected control cultures. A similar effect is seen when cultures are sham-treated with a glutamate-free wash. However, when endogenous NMDA receptor activation is blocked, supplementing the growth medium with 100 µM 2-APV, no decrease in survival is seen in neurons transfected with p600P. *, p < 0.05; **, p < 0.01; N.S., p > 0.05 (see values and statistics under “Results”). Error bars, S.E.
2.5 Discussion

Although chronic neurodegenerative diseases are thought of as slow progressive disorders, the final steps resulting in cell death are remarkably similar to those described in acute neuronal degeneration (1–7). In both cases, influx of Ca\(^{2+}\) and disruption of the cytoskeleton compromise neuronal integrity, yet the Ca\(^{2+}\) signaling roles of cytoskeletal proteins remain largely unexplored. On the basis of the mechanistic similarities between chronic and acute neurodegeneration and the dual CaM-binding and microtubule-stabilizing roles of p600, we explored p600 as an interface for these roles in neuronal pathology. However, p600 does not promote neuronal survival in response to glutamate-induced Ca\(^{2+}\) influx through its microtubule-stabilizing function, as evidenced by the ineffectiveness of paclitaxel in preventing Ca\(^{2+}\) dyshomeostasis after p600 depletion (Table 2.1). The neuronal death observed upon p600 depletion (Figure 2.1E) is preceded, within 5 min of glutamate treatment, by a rapid Ca\(^{2+}\)-induced dyshomeostasis characterized by rapid aggregation of GFP-CaMKII\(\alpha\) and fragmentation of the ER. Interestingly, the close temporal link between Ca\(^{2+}\)-induced CaMKII aggregation and fragmentation of the ER is also seen in smooth muscle cells with the \(\delta\) isoform of CaMKII, where the aggregation is also thought to be indicative of Ca\(^{2+}\) overload (41). Of note, the observed aggregation of CaMKII\(\alpha\) is distinct from the localization of CaMKII to microtubules upon local NMDA stimulation, which can be blocked by KN-93 (42). It is also distinct from the other pathological localization of CaMKII to the postsynaptic density, which can be blocked by KN-62 (43) (reviewed in Ref. 44). This finding, following previous reports of CaMKII\(\alpha\) aggregation in pathological circumstances (26–32), lends support to our analysis of CaMKII\(\alpha\) aggregation as a biomarker for early Ca\(^{2+}\)-induced neuronal dyshomeostasis. Using pharmacological and molecular approaches, we delineate a path whereby glutamate stimulation...
Figure 2.6. p600 prevents neuronal death in response to glutamate-induced cytosolic Ca^{2+} elevation. Left, Ca^{2+} influx via NMDARs or presumptive mGluR-activated endoplasmic reticulum Ca^{2+} efflux via IP3 receptors causes p600 to complex with CaM and CaMKIIα, promoting neuronal survival. Right, disruption of the p600/CaM interaction or depletion of p600 by RNAi causes a rapid aggregation of CaMKII, fragmentation of the endoplasmic reticulum, and ultimately neuronal death.
of NMDARs and activation of ER IP3 receptors (via presumptive metabotropic glutamate receptor activity) allow Ca$^{2+}$ entry, which causes p600, CaM, and CaMKIIα to form a complex mediated by a direct p600/CaM interaction (summarized in the left panel of Figure 2.6). The p600/CaM interaction was found to be atypical in its stoichiometry, and p600P has no primary sequence homology elsewhere in the proteome. Hence, p600P is probably extremely specific to the p600/CaM interaction and thereby useful as a tool to specifically disrupt the p600/CaM interaction. It is worthwhile to note that Figure 2.4D shows a basal interaction (lane 1) between p600 and CaM, which is enhanced in the presence of Ca$^{2+}$ (lane 2) and unaffected by the blocking peptide (lane 3) but disrupted by both together (lane 4). This observation presaged our conclusion that the effects deriving from the p600/CaM interaction are Ca$^{2+}$-dependent. Figure 2.5 shows that the direct p600/CaM binding is required to prevent the GFP-CaMKIIα aggregation (A) for survival (B), indicating that in the normal neuron, p600 promotes survival by a Ca$^{2+}$-dependent binding to calmodulin. NMDAR blockade prevented both GFP-CaMKIIα (Table 2.1) and cell demise under ambient activity (Figure 2.5B), further indicating mutual cause. Importantly, the unexpected finding that the brief 32min dose of glutamate used in the analyses of rapid perturbations to neuronal homeostasis (Figure 2.2 and Figure 2.5A) experiments had no detectable effect 24 h later in cell death experiments (Figure 2.5B, left and center) was an important validation of the experimental design; a 30 µM dose of glutamate, although required to show a difference in CaMKIIα aggregation minutes later, was insignificant compared with 24 h of ambient activity in these cultures. The rescue of cell death by blockade of ambient NMDAR Ca$^{2+}$ influx (Figure 2.5B, right) further proves that p600 promotes survival at ambient (subpathological) levels of neuronal activity. The lack of observed differences in CaMKIIα aggregation (Figure 2.5A) or base-line CaMKIIα phosphorylation (Figure 2.1C)
without 24-h incubation probably reflects a less complete knockdown and a lower summation of spontaneous activity. This study opens a line of questioning pertaining to the short term significance of the p600/CaM interaction; although this interaction is certainly required for survival, it is unknown what events transpire to promote survival or to cause death in the absence of the p600/CaM interaction. p600 appears not to counteract the microtubule-destabilizing effects of CaM because paclitaxel did not prevent CaMKIIα aggregation after glutamate treatment. That said, our data reported here suggest that p600 promotes survival entirely by transducing Ca\(^{2+}\) signals. Importantly, the base line of CaM/CaMKIIα signaling is not altered in the absence of p600 because no difference was observed in the levels of phosphothreonine 286-CaMKIIα (activated CaMKIIα) between control RNAi and p600-depleted RNAi neurons (Figure 2.1C). The significance of the p600/CaMKIIα interaction (Figure 2.3) remains to be determined, although p600 is one of many cytoskeletal proteins with which CaMKII associates (see Ref. 45 for a review). The inability of treatment with KN-62 and KN-93, inhibitors of the CaM/CaMKII interaction, to rescue neurons from Ca\(^{2+}\)-induced dyshomeostasis also demonstrates that p600 promotes survival by pathways that do not involve classical CaM-dependent CaMKIIα activation. Neuronal survival seems to rather be dependent on the Ca\(^{2+}\)-dependent p600/CaM interaction. A probable explanation is that the p600/CaM interaction forms a feedback mechanism that inhibits further Ca\(^{2+}\) influx following abnormally high bouts of glutamatergic stimulation, although this possibility remains untested. The relationship between the findings that p600 is an ER- and microtubule-associated protein (15) and the Ca\(^{2+}\)-dependent function reported here is similarly unclear, and it remains a possibility that there are multiple subcellular pools of p600 with distinct functions. Perhaps the most conspicuous question remaining is the precise relationship between the rapid indicators of Ca\(^{2+}\) dyshomeostasis, namely the
fragmentation of the ER and aggregation of CaMKIIα, and neuronal demise hours later. Although these analyses provide enticing detail of mechanisms of Ca\(^{2+}\) dyshomeostasis, we cannot conclude that such mechanisms are more than mere correlates of the neuronal demise we separately quantify. Accordingly, the conclusions drawn by this study are conservative and derived entirely from direct assessment of neuronal demise (especially in Figure 2.1E and Figure 2.5B), whereas our analysis of Ca\(^{2+}\)-induced neuronal dyshomeostasis (Figure 2.2 and Table 2.1) is included in order to provide the reader with mechanistic insight. We must also make clear that the aggregation of CaMKIIα and the fragmentation of the ER are used here as markers of cellular dyshomeostasis rather than markers of cell death. The question of the precise manner of neuronal demise is likewise still open. We used only mild concentrations of exogenous glutamate and even observed NMDAR-dependent death under ambient culture treatment, making necrotic neuronal death unlikely (46). Thus, although we observed necrotic cells in our cultures, depletion of p600 by RNAi did not increase their frequency (Figure 2.1E). We also found no apoptotic indicators following depletion of p600 by RNAi (Figure 2.1E) and similarly no delayed apoptosis over the 24 h after which we saw neuronal death when we blocked the p600/CaM interaction. Because p600 is an ER-associated protein (15), ER stress pathways may be activated (47, 48). We can, however, rule out ER stress-induced apoptosis (49) based on the failure of the pancaspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone to rescue neuronal demise (see “Results”). Direct inhibition of ER stress with the chemical chaperone and ER stress inhibitor 4-PBA was similarly ineffective at preventing both neuronal demise and the aggregation of CaMKIIα. These data together absolve ER stress as a causal factor in the neuronal demise following disruption of p600 function. However, a closely related possibility is that p600 is required for autophagy and/or protein degradation-dependent survival. Broadly speaking,
neurons can die by necrosis, apoptosis, or autophagy. Because we were unable to detect signs of the former two mechanisms, we are bound to suspect the latter. This suspicion is well grounded in the published role of p600 in the N-end rule pathway (50) and especially in new work (24) showing that p600 knock-out mice die during embryogenesis, with yolk sac development defects characterized by aberrant autophagy (elaborated in Ref. 51). Another recent analysis of a second distinct p600 knock-out mouse strain found severe defects in the embryonic heart but also found no indications of apoptosis (25). Unfortunately, analysis of the autophagic process or defects therein is complex and well beyond the scope of this study. In fact, the role of calcium in the induction of autophagy, or even the role of autophagy in cell death, are not completely understood in any system (52, 53). However due to its established role in protein degradation pathways and its role in Ca^{2+} signaling presented in this study, p600 is an excellent candidate molecule for a link between Ca^{2+} signaling, autophagy, and neurodegeneration. Although excessive cytosolic Ca^{2+} normally bodes ill for hippocampal neurons, p600 is in a unique position to promote survival via a Ca^{2+}/CaM-dependent interaction. The prevalence of p600, CaM, and CaMKIIα in neurons susceptible to excitotoxicity further suggests a role for p600 in the array of acute and chronic degenerative neuropathologies in which Ca^{2+} dysregulation in neurons plays a central role.

2.6 References


[43] F. Meng, J. Guo, Q. Zhang, B. Song, and G. Zhang, “Autophosphorylated calcium/calmodulin-dependent protein kinase II alpha (CaMKIIalpha) reversibly targets to


Chapter Three: p600 stabilizes microtubules to prevent the aggregation of CaMKIIα during photoconductive stimulation

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3.1 Abstract

The large microtubule-associated/Ca^{2+}-signalling protein p600 (also known as UBR4) is required for hippocampal neuronal survival upon Ca^{2+} dyshomeostasis induced by glutamate treatment. During this process, p600 prevents aggregation of the Ca^{2+}/calmodulin-dependent kinase IIα (CaMKIIα), a proxy of neuronal death, via direct binding to calmodulin in a microtubule-independent manner. Using photoconductive stimulation coupled with live imaging of single neurons, we identified a distinct mechanism of prevention of CaMKIIα aggregation by p600. Upon direct depolarization, CaMKIIα translocates to microtubules. In the absence of p600, this translocation is interrupted in favour of a sustained self-aggregation that is prevented by the microtubule-stabilizing drug paclitaxel. Thus, during photoconductive stimulation, p600 prevents the aggregation of CaMKIIα by stabilizing microtubules. The effectiveness of this stabilization for preventing CaMKIIα aggregation during direct depolarization but not during glutamate
treatment suggests a model wherein p600 has two modes of action depending on the source of cytosolic Ca\(^{2+}\).

### 3.2 Introduction

Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) accounts for nearly 1% of the total protein in the mammalian brain [1]. By virtue of its toroidal arrangement, its subunits are able to phosphorylate one another, entering into a sustained period of kinase activity. This molecular hysteresis and accompanying signalling activity has led to the hypothesis that CaMKII is a medium of Ca\(^{2+}\)-dependent memory [2]. The localization and activity of CaMKII is tightly linked to Ca\(^{2+}\)-mediated neuronal survival. Under ischemic conditions in vivo, CaMKII rapidly enters a particulate state wherein it loses kinase activity for a prolonged period [3–5]. Experimental reproductions of the lowered pH, uncoupled oxidative phosphorylation, and Ca\(^{2+}\) overload characteristic of the ischemic brain induce the α isoform of CaMKII (CaMKIIα) to form similar long-lasting self-aggregates [6–10]. While these various reports of pathological inactivation and self-aggregation are likely observations of the same phenomenon, this is still uncertain, as is the role of this pathological behaviour. Furthermore, not only does CaMKII respond to Ca\(^{2+}\) influx, its activation state also controls it: CaMKII inhibition causes sufficient glutamate-induced Ca\(^{2+}\) entry to cause neuronal death under ambient culture activity [11]. Thus, studies of CaMKII, with its extraordinarily high neuronal expression, its role in synaptic plasticity and memory, and its direct regulation of neuronal survival, are of paramount importance as proxies of neuronal well-being and outcome.

p600 (or UBR4) is a large multifunctional protein (600 kDa) that plays an important role in neurite outgrowth, neuronal migration, protein degradation, Ca\(^{2+}\) signalling, cell adhesion, cell
survival, and autophagy [12–16]. p600 is enriched in the brain, particularly in neurons, and has at least one microtubule-stabilizing domain [12]. We recently reported that depletion of p600 by RNAi significantly increases the proportion of neurons showing CaMKIIα aggregation upon glutamate-induced Ca\(^{2+}\) entry in hippocampal cultured neurons [17]. p600 was found to form a complex with CaM and CaMKIIα, mediated by a direct and atypical interaction between p600 and CaM. Specific disruption of this interaction resulted in neuronal death under ambient activity, and potentiated CaMKIIα aggregation following application of mild doses of exogenous glutamate. Using an array of inhibitors, it was determined that the primary contributors to CaMKIIα aggregation were Ca\(^{2+}\) influx through N-methyl-D-aspartate receptors (NMDA) and metabotropic release of Ca\(^{2+}\) through inositol 1,4,5-trisphosphate receptors. Importantly, pre-incubation with the CaMKII inhibitors KN62 or KN93 failed to prevent the aggregation of CaMKIIα, demonstrating that the aggregation was CaM-independent and distinct from the translocation to synapses [18]. Furthermore, stabilization of microtubules with paclitaxel had no effect, demonstrating that p600 did not prevent CaMKIIα aggregation via its microtubule stabilizing function. Intriguingly, inhibition of voltage-gated L-type Ca\(^{2+}\) channels modestly rescued the aggregation of CaMKIIα. This suggests that p600 could modulate CaMKIIα aggregation upon direct depolarization. To further investigate this finding, we analyzed the spatio-temporal dynamics of CaMKIIα aggregation induced by photoconductive stimulation, a technology that allows for the direct depolarization of single neurons coupled with live imaging.
3.3 Materials and methods

3.3.1 Cell culture, transfection, and photoconductive stimulation

Dissociated cultures of rat primary hippocampal neurons were prepared on silicon wafers for photoconductive stimulation as described previously [19, 20]. All animals were housed and handled according to Canadian Council on Animal Care guidelines and experimentation approved by the Health Sciences Animal Care Committee. Neurons were co-transfected with GFP-CaMKIIα and either control or p600 RNAi vectors (molar ratio 1:2) at DIV10 [17]. The day following transfection, silicon wafers were mounted in a specialized photoconductive stimulation apparatus [20] and immersed in a pre-warmed bath solution consisting of 135 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 3 mM CaCl₂, 10 mM d-glucose, and 10 mM HEPES (pH 7.3). For trials with paclitaxel, neurons were pre-incubated for 15 min with 100 nM paclitaxel (Sigma) or a DMSO vehicle (1:10000). Individual neurons were selected which expressed GFP-CaMKIIα. Under constant light intensity, the neurons were depolarized with a 4 V square wave at 100 Hz, 5 ms pulse duration. High-resolution images were captured at ~1.4 frames per second, using a 100x water-immersion objective on an Olympus BX61WI microscope and a Watec N102 integrating CCD camera.

3.3.2 Quantification, curve fitting and statistical analysis

Neurons were depolarized and 5 min later categorized as displaying GFP-CaMKIIα aggregation or not (Figure 3.1). Neurons that did not show GFP-CaMKIIα aggregation after the initial depolarization were depolarized for 30 s to validate the experimental setup, and those that did not show GFP-CaMKIIα aggregation after that were not used for curve fitting or statistical analysis. The data presented in Figure 3.1 are fit to a Boltzmann distribution. For accurate statistical
analysis, the data were treated as discrete. The mean and standard error were determined by iteratively fitting a cumulative mass function to the data. The results were used to compare the datasets using Student’s t test.

### 3.3.3 Quantification of single-cell aggregation

Using ImageJ, the degree of aggregation of GFP-CaMKIIα was determined in each frame of the video by taking intensity thresholds. The automatic particle analyzing function was then used to count the average number of particles. 38 different intensity thresholds were used covering the range of intensities over which the image of the soma was not saturated. The results are plotted in the graph in Figure 3.2C.

### 3.3.4 Dendrite analysis

In Figure 3.2D, the images are brightness and contrast-adjusted to clearly show the localization of the aggregates. The trace in the final panel was created using the threshold-adjusting function of ImageJ, wherein the threshold was adjusted to show the initial synaptic distribution of GFP-CaMKIIα. The outline was then automatically traced and overlaid on the final frame for comparison.

### 3.4 Results

#### 3.4.1 Depletion of p600 lowers the threshold of GFP-CaMKIIα aggregation following photoconductive stimulation

Our previous findings showed that inhibiting L-type Ca$^{2+}$ channels had a significant effect in preventing CaMKIIα aggregation following bath application of glutamate and glycine [17]. Based on that, we assessed if direct depolarization of single neurons could cause CaMKIIα
aggregation. We constructed a dose-response curve wherein single neurons were depolarized by photoconductive stimulation [19, 20] for a given time, incubated for an additional 5 min, then scored for the presence or absence of GFP-CaMKIIα aggregates.

When transfected with a control RNAi, neurons formed aggregates after an average of 9.49 ± 0.24 s (± SEM, n = 78) of depolarization. We then assessed if knockdown of p600 would potentiate this effect. When p600 is knocked down by RNAi, neurons form aggregates after an average of only 1.77 ± 0.36 s (n = 50), resulting in a left-shifted dose-response curve (t₃ = 17.7, p < 0.001 by Student’s t test). Thus, the absence of p600 predisposes neurons to undergo CaMKIIα aggregation after direct depolarization.
Figure 3.1. p600 reduces the length of depolarization required to cause neurons to enter a state wherein GFP-CaMKIIα is aggregated. Neurons were depolarized for the indicated time, allowed 5 min to respond, and scored for the presence or absence of GFP-CaMKIIα aggregation. When fit to a Boltzmann distribution and compared against one another, the data show that depletion of p600 by RNAi causes a mean 80% reduction in the length of depolarization required to cause GFP-CaMKIIα to aggregate (control RNAi, n = 78 neurons; p600 RNAi, n = 50 neurons; \( t_3 = 17.7, p < 0.001 \) by Student’s t test).
3.4.2 Depletion of p600 interrupts the cytoskeletal localization of GFP-CaMKIIα with irreversible aggregation

A recent study showed that during KCl depolarization, CaMKIIα undergoes a rapid and sustained translocation to microtubules [21]. Since the microtubule translocation and self-aggregation of CaMKIIα can both be caused by depolarization, but are mutually exclusive behaviours, we sought to compare the time-course and localization of GFP-CaMKIIα during neuronal depolarization. We co-transfected single neurons with GFP-CaMKIIα and control or p600 RNAi, depolarized them at 0 s (for 5 s) and filmed for 5 min.

We live-imaged 9 control RNAi-transfected neurons and 11 p600 RNAi-transfected neurons. Figure 3.2 shows representative examples. Figure 3.2A depicts a neuron co-transfected with control RNAi wherein GFP-CaMKIIα is at first relatively diffuse, but within 1 min, it translocates to the microtubules. This initial translocation is largely sustained for the remainder of the experiment.

By contrast, a neuron transfected with p600 RNAi (Figure 3.2B) shows the same translocation of GFP-CaMKIIα to the microtubules, but this process is ostensibly less pronounced, and is interrupted by a sustained aggregation of GFP-CaMKIIα. The degree and time-course of aggregation of these representative neurons is quantified and plotted below. Figs 1 and 2 together show that there is a duration of depolarization after which the microtubule translocation of GFP-CaMKIIα is interrupted by self-aggregation, and that p600 sets the threshold at which the former behaviour transitions to the latter.
3.4.3 Stabilization of microtubules rescues the aggregation of GFP-CaMKIIα after direct depolarization

p600 directly binds and stabilizes microtubules [12]. We hypothesized that neurons in which p600 is depleted might have a less stable population of microtubules, making the translocation of GFP-CaMKIIα to these microtubules less probable. By replacing the microtubule-stabilizing function of p600 with 100 nM paclitaxel, we were able to significantly decrease the proportion of neurons showing GFP-CaMKIIα aggregation. At 5 s of depolarization, only 37.5% of neurons with p600 knockdown showed aggregation (6/16 with 100 nM paclitaxel, 37/37 with vehicle control 0.01% DMSO; p = 0.002 according to the two-tailed Fisher’s exact test). We conclude that during direct depolarization, p600 prevents GFP-CaMKIIα aggregation by stabilizing microtubules.

3.4.4 GFP-CaMKIIα aggregation occurs primarily in the dendritic shaft

Lastly, we sought to compare the spatial dynamics of the CaMKIIα aggregation seen here to the synaptic translocation of CaMKIIα [18]. We therefore took advantage of our live-imaging model to take high-resolution footage of the primary dendrite during depolarization. Figure 3.2D shows that during depolarization, the aggregation of GFP-CaMKIIα takes place primarily in the dendritic shaft. The overlay (on the final panel, in white) of the initial distribution of GFP-CaMKIIα over the final aggregated distribution illustrates that the aggregation involves a net (though incomplete) evacuation of dendritic protrusions. While we cannot conclude that the aggregation takes place exclusively outside of the synapses, we can reasonably claim the aggregation of GFP-CaMKIIα seen in this study is not a synaptic phenomenon.
Figure 3.2. In the absence of p600, the cytoskeletal localization of GFP-CaMKIIα is interrupted by a long-lasting self-aggregation. These are representative images. A and B – In a neuron transfected with control RNAi, upon depolarization, GFP-CaMKIIα undergoes a rapid and sustained translocation to the microtubules. When p600 is depleted, the cytoskeletal translocation of GFP-CaMKIIα is interrupted by a sustained aggregation. C – Frame-by-frame quantification of these movies shows that the aggregation of GFP-CaMKIIα takes place between 1 and 5 min after depolarization. D – Imaging of a single primary dendrite shows that the aggregation of GFP-CaMKIIα takes place primarily in the dendritic shaft.
3.5 Discussion

These data suggest a model wherein CaMKIIα translocates to microtubules during induced depolarization, and with a longer depolarization it begins to self-aggregate. In this model, p600 stabilizes microtubules in order to set the threshold at which CaMKIIα switches from microtubule translocation to self-aggregation. The notion that p600 regulates the localization of CaMKIIα by stabilizing microtubules is novel, and complements our previous study showing that p600 prevents CaMKII aggregation through binding to CaM in a microtubule-independent manner [17]. The effectiveness of microtubule stabilization for preventing CaMKIIα aggregation during direct depolarization, but not during glutamate treatment, led to a model wherein p600 has two modes of action depending on which channels are activated.

Based on the conservative assumption that the aggregation we have observed here is driven by Ca$^{2+}$, as it has been in every experimental paradigm so far, there are two possible mechanisms by which the microtubule-associated function of p600 could prevent CaMKIIα aggregation. The first general model is that the concentration of cytoplasmic Ca$^{2+}$ is unaffected by the actions of p600, and that the response of CaMKII to the same stimulus is altered. Based on the interaction between p600 and CaMKIIα and the ubiquitous intracellular expression of both [17], it is possible that during voltage-dependent Ca$^{2+}$ influx CaMKIIα would normally target to p600 or to the stable microtubules it sustains. Depletion of p600 could remove these docking sites and thereby favour the self-aggregation of CaMKIIα.

The second possibility is that p600 regulates Ca$^{2+}$ influx. Furukawa and Mattson [22] demonstrated that the stabilization of microtubules by paclitaxel can in fact restrict excitotoxic Ca$^{2+}$ entry, and that microtubule destabilization causes neuronal death. Thus in addition to more well-known mechanisms by which cytosolic Ca$^{2+}$ affects microtubule stability [24, 23],
microtubule stability in turn affects levels of cytosolic Ca$^{2+}$. By analogy to that study, p600 may stabilize microtubules during direct depolarization, restricting Ca$^{2+}$ entry and preventing CaMKII$\alpha$ aggregation. Though Furukawa and Mattson did not find that Ca$^{2+}$ entry by KCl depolarization was mitigated by paclitaxel pre-treatment, they did find that the effect of microtubule stabilization on Ca$^{2+}$ entry was channel-specific. This precedent of channel-specificity could offer means to reconcile the effectiveness of microtubule stabilization in direct depolarization that was not found after glutamate/glycine treatment.

The finding that p600 acts differently to prevent CaMKII$\alpha$ aggregation when faced with different sources of Ca$^{2+}$ strongly suggests a heterogeneous subcellular response by p600. Efforts to distinguish between these two general models will begin with detailed measurements of local cytosolic Ca$^{2+}$ concentrations, and will require minute observations of microtubule dynamics when p600 is depleted.

It is noteworthy that the aggregation of CaMKII$\alpha$ is distinct from its translocation to synapses [18]. In this report, the aggregation takes place both in the soma and the dendrites (Figure 3.2B and D), as opposed to the purely synaptic targeting seen in the former case. Interestingly, the endogenous CaMKII inhibitor, CaMKII-N, resides exclusively in the dendritic shaft and soma [25]. Apart from the movement away from the synapses and into the dendritic shaft, there is a case for the inactivation of CaMKII. Firstly, the in vitro and in vivo inactivation of CaMKII follows a similar time-course with a completion time of about 5 min, which has been closely correlated to the aggregation and movement to the particulate fraction [4, 6, 26]. This time-course of inactivation and aggregation is strikingly similar to the aggregation quantified in Figure 3.2C. Secondly, our previous work has shown that the depletion of p600 or disruption of the p600/CaM interaction not only causes CaMKII$\alpha$ aggregation, but also causes neuronal death.
Since CaMKII tends to both inactivate and aggregate in excitotoxic conditions [4], the ultimately fatal outcome of p600 depletion suggests that the aggregation of CaMKIIα seen in this study also involves an inactivation.

Though the purpose of the CaMKIIα aggregates remains unknown, entry into this state can reasonably be supposed to preclude other physiological behaviours of CaMKIIα. Even allowing for the slight possibility that CaMKIIα in its aggregated form is able to interact with its usual binding partners, we can assume that these interactions would be greatly diminished on account of its sequestration in aggregates. For example, CaMKII directly phosphorylates the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor in order to potentiate synaptic strength [28, 27]. The implied inactivation of its kinase activity, the reduced mobility/larger size of the aggregates, and the net synaptic evacuation we observed (Figure 3.2D) combine to make it unlikely that CaMKIIα aggregates are able to phosphorylate the AMPA receptor in any significant way. Similarly, aggregated CaMKIIα is unlikely able to maintain its usual role in binding and modulating the properties of NMDA receptors and L-type channels [29, 30]. The combined evidence strongly suggests that in its aggregated form, CaMKIIα is unable to continue in its role as a modulator of Ca^{2+}-signalling and synaptic plasticity. As a direct regulator of CaMKIIα aggregation, p600 is thereby a hypothetical regulator of these processes.

The consequence of interrupting the microtubule binding of CaMKIIα is more difficult to predict since it is not fully clear what function(s) the CaMKIIα/microtubule interaction serves. CaMKII, predominantly the α-isoform, associates with microtubule preparations, though it is not clear to what element of microtubules it binds [31, 32]. However, it has been shown that CaMKIIα directly phosphorylates α-tubulin near the C-terminus [31, 32], thereby favouring the
depolymerisation of microtubules [32]. This association and phosphorylation has even been proposed to be a means of directly encoding information on microtubules [33]. The only study of the functional consequences of the CaMKIIα/microtubule interaction [21] offered strong evidence that an interaction between CaMKIIα and dendritic microtubules increases surface expression of AMPA receptors and spine remodelling. This study also showed that this translocation of CaMKIIα requires stable microtubules [21]. Although the intermediate mechanism is speculative, their result supports the model wherein activated CaMKIIα translocates to microtubules to ultimately potentiate synaptic strength. Therefore, p600 could be a regulator of synaptic strength by regulating the transition between the microtubules translocation and aggregation of CaMKIIα.

While CaMKIIα aggregation can be experimentally induced by all manner of pharmacological insults, the depletion of p600 or the mere disruption of the p600/calmodulin interaction [17] remains the only known means of inducing a neuron to enter this state by its own activity. Not only does p600 regulate the localization of CaMKIIα, p600 is also required for neuronal survival [17]. While a general understanding of the role of CaMKIIα aggregation in neurons remains elusive, it is a topic that warrants further study both because of the importance of the enzyme itself, and as a proxy of neuronal death. This study also offers the first indication that the self-aggregation CaMKIIα is dependent on microtubule stability, and it introduces p600 as the mediator of this effect. Since Ca²⁺ overload, microtubule instability and p600 depletion have all been independently shown to cause neuronal death [17, 34, 35], this study puts us closer to a unified model wherein p600 is an interface between microtubule dynamics, Ca²⁺ signalling, and neuronal survival.
3.6 References


Chapter Four: General Discussion

Together, the two manuscripts have first established that p600 is a required for neuronal survival by forming a direct interaction with CaM, and that it enters into a tripartite p600/CaM/CaMKIIα complex. Blocking the p600/CaM interaction is sufficient to cause Ca\(^{2+}\)-dependent neuronal death over the course of 24 hours of ambient Ca\(^{2+}\) entry via the NMDA receptor. Upon induced Ca\(^{2+}\) entry, neurons rapidly enter a state wherein the ubiquitous hippocampal kinase CaMKIIα enters an aggregated state indicative of pathological intracellular conditions. The use of this aggregation as a marker allowed the delineation of the pathways of Ca\(^{2+}\) elevation when p600 was depleted, and to determine that p600 acts by stabilizing MTs depending on the source of Ca\(^{2+}\) entry. The foremost unanswered question of this thesis concerns the relationship between the rapid aggregation of CaMKIIα, and the eventual neuronal death. Section 4.1 is therefore dedicated to a discussion of this question. Section 4.2 expounds on the mechanism of p600 function put forth in Chapters 2 and 3, offering theories explaining the regulation of neuronal survival by p600 and detailing experiments to test those theories.

4.1 The relationship between CaMKIIα aggregation and neuronal death

The data presented in this thesis present two possible accounts of the events between the formation of CaMKIIα aggregates and neuronal death. The first is that the two outcomes are unrelated, and originate separately from a pathological Ca\(^{2+}\) elevation. This possibility, discussed in section 4.1.1, would indicate that the p600/CaM/CaMKIIα complex is part of a negative-feedback system for the elevation of cytosolic Ca\(^{2+}\), to which CaMKIIα aggregation and neuronal death are autonomous responses. The second possibility is that p600 plays a direct role in preventing CaMKIIα aggregation, which is taken to be in itself a neurotoxic stimulus. The
evidence for a causal structure between CaMKIIα aggregation and neuronal death is discussed in section 4.1.2.

4.1.1 p600 as a Ca\(^{2+}\)-dependent regulator of Ca\(^{2+}\) elevation

When the p600/CaM interaction is blocked neurons undergo CaMKIIα aggregation and later die, and both processes are dependent on Ca\(^{2+}\) entry through the NMDA receptor. Both processes could therefore be adequately explained by the hypothesis that in response to Ca\(^{2+}\) entry through the NMDA receptor, the p600/CaM interaction regulates further Ca\(^{2+}\) entry into the cytosol. This model is strongly supported by previous studies showing that purified CaMKIIα will aggregate [137], [138], and that aggregates from neurons can be purified to homogeneity [143]: Aggregation therefore requires no external influence once the conditions of an elevation of cytosolic Ca\(^{2+}\) and a drop in pH are met. Additionally, the kinetics of in vitro aggregation to that in cells is so similar [136], [137], [139] as to suggest that the entry into an aggregated state in neurons is not catalyzed or regulated by neuronal proteins. This theory may be easily tested by transfecting neurons with p600P and treating with Glu / Gly while simultaneously using a Ca\(^{2+}\)-sensitive fluorophore to measure cytosolic Ca\(^{2+}\) levels. For this purpose, the canonical ratiometric dye Fura-2 would suffice as it is sensitive in the resting physiological range of neurons and therefore qualified to show deviations therefrom [151]. Neurons transfected with p600P ought to show a runaway Ca\(^{2+}\) signal, or one that is slower to dissipate, characteristic of a failed negative-feedback system. If detected, the two plausible sources for such a cytosolic Ca\(^{2+}\) elevation are the extracellular space and the ER.
4.1.1.1 p600 as a regulator of external Ca$^{2+}$ entry

p600 dysfunction must account not only for eventual neuronal death, but also for the rapid aggregation of CaMKIIα, which begins about a minute after the stimulus, and is complete after about 5 minutes. This rapid action precludes *de novo* translation of Ca$^{2+}$ channels, but would allow for the expression of already-translated channels. AMPA receptors, which are known to be inserted into the plasma membrane during synaptic plasticity in a time frame of 1-5 minutes [152], [153], are not involved as evidenced by the ineffectiveness of CNQX in blocking the development of CaMKIIα aggregation (Table 2.1). The time frame also allows for a phosphorylation event. The NMDA receptor itself has over 20 potential phosphorylation sites that modify everything from its current to its synaptic localization (reviewed in [154]). It would be futile to offer speculations as to which of these sites might be involved, but given that the NMDA receptor is so well-poised to allow for excitotoxic Ca$^{2+}$ entry (see section 1.2.1.1), the role of p600 may be to suppress an activity-enhancing phosphorylation event on the NMDA receptor. It would furthermore be informative to assess the difference in the subunit composition of the NMDA receptor. In the adult hippocampus, NMDA receptors are composed of both NR2A and NR2B subunits [155], which are thought to promote pro-survival and pro-death signalling pathways respectively [156]. However in DIV 10 hippocampal cultures such as those used in the experiments detailed in Chapters 2 and 3, NMDA receptors are composed of mostly NR2B subunits [157]. Unlike in neurons of the fully developed hippocampus, these NR2B-containing receptors are thought to promote both pro-death and pro-survival signalling [157]. p600 may therefore act by promoting pro-survival signalling through the minority of NR2A-containing receptors or the larger population of NR2B-containing receptors, or by inhibiting the pro-death signalling initiated by NR2B-containing receptors. An effect of p600 on NMDA receptor
activation would therefore have to be specific to the NR2A or NR2B subunit. Selective antagonists against either NR2A- or NR2B-containing receptors, or genetic ablation of either receptor type, would be required to determine which is required for the pro-survival effect of p600.

4.1.1.2 p600 as a regulator of ER Ca\(^{2+}\) store release

The contribution of ER Ca\(^{2+}\) reserves to CaMKII\(\alpha\) aggregation in p600-depleted neurons was demonstrated by the inhibitory effect of the IP3R blocker 2-APB. While 2-APB is adequately specific to claim that its block of an immediate and demonstrably Ca\(^{2+}\)- and glutamate-dependent effect was due to Ca\(^{2+}\) efflux from IP3Rs, neither it nor any other IP3R blocker is suitable for the 24 hour blockade required to demonstrate a relief of p600P2-induced neuronal death (see [158] for a discussion of the specificity of 2-APB). Interestingly, the NMDA antagonist MK801 blocked the entirety of CaMKII\(\alpha\) aggregation, while 2-APB also had a highly significant effect (Table 2.1). It cannot therefore be the case that the NMDA and IP3 receptors are independent contributors to the elevation in cytosolic Ca\(^{2+}\), but rather co-dependent. For example, mGluR activation mediates an increase in glutamate release into the culture medium [159], allowing the possibility of a positive-feedback loop wherein NMDA receptors are further activated. More directly, group I (i.e. postsynaptic [81]) mGluR activation potentiates NMDA receptor activity by a mechanism involving a rise in intracellular Ca\(^{2+}\) [160]. This potentiation is blocked by actin destabilization [160], implicating cytoskeletal regulation of the NMDA receptor or the ER. Since p600 is a determinant of ER morphology (Figure 2.2D,E), one might suppose that role of p600 is to mitigate the excitotoxic contribution of ER Ca\(^{2+}\) through IP3Rs, perhaps by acting through the actin cytoskeleton. The IP3R shows a biphasic Ca\(^{2+}\) release in response to IP3 [161], probably by
directly binding to CaM [162]. p600 may thereby directly regulate the IP3R. Such a role for p600 would an important area of study in that this mechanism would be transferable to non-excitable cells, meaning that p600 may play a role in regulating cytosolic Ca$^{2+}$ in a diversity of cell types.

4.1.2 p600 as a regulator of the neurotoxic aggregation of CaMKIIα

4.1.2.1 Evidence for a causal structure between the aggregation of CaMKIIα and neuronal survival

The work presented in this thesis is the first direct demonstration that CaMKIIα aggregation is a prelude to neuronal death. There is however a matter yet to be settled, to answer whether CaMKIIα aggregation is part of a protective response initiated by the neuron, or a part of a death pathway. Given that inactivation is the principal, and perhaps only, characteristic of aggregated CaMKIIα, comparisons with the effects of direct CaMKII inhibition are enlightening on this point. The newest developments in this area have shown that CaMKIIα is no idle spectator in excitotoxic death: CaMKII inhibition in cortical cultures immediately after an excitotoxic insult is protective [163]. Paradoxically, a protracted (24 hour) inhibition both sensitizes neurons to excitotoxicity [163] and is itself neurotoxic [164]. On a shorter time-scale, CaMKII inhibition causes a rapid increase in neuronal excitability, a rise in glutamate in the culture medium, and Ca$^{2+}$ deregulation in cortical neurons [164]. The later lethality of CaMKII inhibition is accordingly prevented by NMDA antagonism [164]. This data offers two narratives that have not yet been reconciled: One in which CaMKII has neurotoxic activities following excitotoxicity, and another in which its activity represses a feedback-loop of neuronal excitability and NMDA-mediated excitotoxicity. Their reconciliation would require a survey of the substrates of CaMKIIα that enact these time-dependent effects. Regardless of which narrative is more relevant
to conclusions about CaMKIIα aggregation, they share the premise that CaMKIIα aggregation is not epiphenomenal to neuronal fate.

The test of the theory that CaMKIIα aggregation directly influences survival would have to be done in neurons, wherein CaMKIIα activity promotes survival. Since purified aggregated CaMKII could not be practically introduced, mutant forms of CaMKIIα would have to be used. Transfected GFP-tagged mutants that do not aggregate, for example T286D, show no aggregation whatsoever, in spite of the fact that they presumably form holoenzymes with endogenous CaMKII [139]. It must therefore be the case that certain transfected mutants have a dominant-negative effect on endogenous CaMKII. Immunocytochemistry against total CaMKII would be required validate this assertion, by demonstrating that endogenous CaMKII does not aggregate in the presence of ectopically-expressed non-aggregating mutants. If this held true, one would hypothesize that neurons transfected with GFP-CaMKIIα T286D, T305/306D, or any other mutant that resists aggregation, would be more resistant to glutamate excitotoxicity. Transfection with these mutants should nullify the increase in excitotoxicity seen in p600 RNAi and p600P-transfected neurons over the course of 22 and 40 hours post-transfection, respectively (using the protocols described in Figure 2.1E and Figure 2.5B).

4.1.2.2 p600 as a direct modulator of CaMKIIα
The data presented in Chapter 2 showed that p600 enters into a complex with CaMKIIα, probably by way of a direct interaction with CaM [165]. This could be accounted for by a model wherein CaMKIIα would normally target to p600, and in its absence it instead aggregates. This model is however troublesome in that it requires the amount of p600 to be comparable to that of CaMKIIα. For example, 2 CaM molecules associate with 1 p600 ([165]; Figure 2.4B), and 1
CaMKIIα binds per CaM [133]: Even if the p600/CaMKII interaction can accomplished by a single p600:CaM:CaMKII linkage, the amount of p600 needed to sequester CaMKIIα amounts to 1 molecule of p600 per CaMKIIα holoenzyme, very nearly a 1:1 weight ratio. Sequestration by p600 would have to be roughly stoichiometric, since even small concentrations of CaMKII will aggregate, albeit somewhat more slowly [137]. Because of the extraordinary prevalence of CaMKIIα, amounting to 2% of total hippocampal protein [128], any mechanism requiring a matching concentration of p600 is improbable. p600 may however act substoichiometrically in order to modulate CaMKIIα, possibly by stabilizing MTs (see section 4.2.2), or by filling a signalling function which renders CaMKIIα unable to aggregate. Site-directed mutagenesis has revealed a number of phosphorylation sites on CaMKIIα which preclude aggregation [139]: By regulating CaMKIIα phosphorylation state, p600 may prevent its aggregation and thus neuronal survival.

4.2 The mechanism by which p600 acts as a Ca^{2+} signalling protein

The role of p600 described in this thesis is a novel pro-survival function in neurons: The mechanisms of Ca^{2+}-dependent death in hippocampal neurons are well-characterized, but Ca^{2+}-dependent survival signalling is barely understood. The mechanisms by which p600 acts to promote survival can therefore not be easily related to existing survival-signalling pathways, though they can be related to the previously established functions of p600 as a MT-stabilizing and ER-associated protein. The evidence for p600 acting in either capacity is discussed in the sections below.
4.2.1 p600 as a regulator of ER structure and function

In spite of its potential importance, very little is known about how or why the ER fragments. The question of why may be related to the immediate fact that fragmentation entails a loss of continuity [166]: That which was formerly free to diffuse through the luminal space becomes spatially restricted. The most obvious effect would be to restrict ER Ca$^{2+}$ release to a small area of the neuron, mitigating a signal that would otherwise spread through a much greater area. The fragmentation of the ER in neurons is repaired within about 30 minutes [167], supporting the notion that the fragmentation is a temporary “damage control” measure. The question of how the ER fragments is also unanswered. Early work in HEK cells showed that an elevation in the concentration of cytosolic Ca$^{2+}$ is sufficient to cause its fragmentation [168]. In neurons, this Ca$^{2+}$ can be from the activity of NMDA receptors [169], though other sources of Ca$^{2+}$ have not been ruled out.

It is unknown how Ca$^{2+}$ induces ER fragmentation. Since ER membranes are drawn along MTs [59], a loss of this tension may be sufficient. Supposing that the tension of the ER is maintained by a MT:linker:ER linkage, the loss of tension may be because of a failure at either end of the linker, or from MT collapse. p600 is an excellent candidate for the role of a Ca$^{2+}$-sensitive linkage between MTs and the ER. The test of this hypothesis will require specific domain-mapping of the ER-associated and MT-associated domains of p600, accomplished either by subcellular fractionation, immunocytochemistry, or both. If these are found, the exogenous expression of either should be sufficient to fragment the ER, provided the region does not contain both domains. Conversely, the overexpression of full-length p600 in neurons ought to mitigate the fragmentation of the ER by providing more substantial ER-MT linkages. Finally, electron microscopy coupled with immuno-gold labelling of neurons ought to show p600 linking
MTs to the ER. p600 is in fact large enough that, if the sample is prepared correctly, it ought to be visible itself. Neurons pretreated with Glu/ Gly prior to fixation and electron microscopy ought to show a loss of the ER-MT linkage. Whether in this case p600 is retained on MTs or on the ER would begin to answer the question of how p600 maintains the structure of the ER.

The fragmentation of the ER can be induced by an elevation of cytosolic Ca\(^{2+}\), but may in turn also control Ca\(^{2+}\) dynamics. p600 may control the amount of Ca\(^{2+}\) release from the ER (as discussed in section 4.1.1.2), or it may regulate its structure in order to control the localization ER Ca\(^{2+}\) release sites. For example, the ER and mitochondria are held in close proximity, and share Ca\(^{2+}\) stores [170]. The low affinity of mitochondrial Ca\(^{2+}\) uptake requires proximity to a local release site [171]. One might therefore hypothesize that the fragmentation of the ER involves the loss of the ER/mitochondrial contact, nullifying the Ca\(^{2+}\)-buffering capacity of mitochondria and allowing ER Ca\(^{2+}\) to instead flow into the cytoplasm. This may have ambiguous effects, such as activation of Ca\(^{2+}\)-dependent cytosolic proteases. This may conversely avoid the activation of mitochondrial death pathways. The loss of ER continuity during fragmentation [166] could therefore affect neuronal fate by limiting ER Ca\(^{2+}\) release. The role of p600 as a regulator of ER morphology may therefore be one way in which it regulates neuronal survival.

### 4.2.2 The role of p600 in stabilizing microtubules in response to selective sources of Ca\(^{2+}\)

The finding that MT stabilization prevents CaMKIIα aggregation in p600-depleted neurons in response to direct depolarization but not Glu / Gly treatment indicates that p600 has dual roles in response to different sources of Ca\(^{2+}\) elevation, and that one of these roles is as a MT-stabilizing protein. Since p600 is known to affect the stability of neuronal MTs [10], p600 may promote
survival by the Ca\(^{2+}\)-dependent stabilization of MTs. This is however not a simple matter to demonstrate, since Ca\(^{2+}\) itself destabilizes MTs, and CaM acts through MT-associated proteins to destabilize MTs [172], [173]. Appendix 3 presents evidence for a direct Ca\(^{2+}\)-dependent stabilization of MTs by p600\(^{3910-4851}\) that seems to counteract the destabilizing effects of Ca\(^{2+}\) (see data and suggested experiments therein). If p600 is indeed found to stabilize MTs in a Ca\(^{2+}\)-dependent manner, it could account for previous work showing that neuronal MT stability is an effector of Ca\(^{2+}\) entry and neuronal survival (see section 1.2.3.2).

4.2.2.1 The Ca\(^{2+}\) source-dependence of microtubule-stabilization by p600

The fact that p600 stabilizes MTs in response to VDCC Ca\(^{2+}\) entry, but not glutamatergic Ca\(^{2+}\) entry, is reminiscent of previous work showing a channel-specific control of Ca\(^{2+}\) influx by MT stabilization [126]. Though this MT control of Ca\(^{2+}\) entry was shown only for AMPA receptors [126], it is possible that a neuron depleted of p600 would show the same effect for other channels, namely the VDCCs activated during direct depolarization. In order to dissociate channel-specific effects, specific ligands would be applied, namely NMDA and Bay K8644 to target NMDA receptors and L-type channels respectively. Since p600 must act within 1-5 minutes of channel activation, based on the kinetics of CaMKII\(\alpha\) aggregation, neurons should be rapidly fixed at the 5 minute mark, and immunostained to assess MT integrity by confocal microscopy, or assessed without immunostaining by electron microscopy. Neurons depleted of p600, or in which the p600/CaM interaction is blocked with p600P, should show fewer stable MTs. Conversely, neurons over-expressing full-length p600 ought to show more MT stability. Based on the effectiveness of paclitaxel in preventing CaMKII\(\alpha\) aggregation in direct
depolarization but not Glu /Gly treatment, one would predict that MTs would show no p600-dependent effects after NMDA treatment, but that they would after Bay K8644 treatment.

4.2.2.2 The hypothetical regulation of microtubule stability by CaMKIIα

Though it is unknown exactly how MT stability affects neuronal survival, it may involve CaMKIIα: A recent study showed that CaMKIIα undergoes an activity-dependent translocation to MTs, and that the destabilization of MTs had a significant effect in interrupting this translocation [174]. Since the endogenous p600 is sufficient to have a demonstrable effect on MT stability [10], p600 may provide a docking site on microtubules for CaMKIIα, in addition to directly binding to CaMKIIα. p600 may also be regulating the phosphorylation of tubulin by CaMKIIα, either promoting phosphorylation to induce MT destabilization, or preventing it to promote MT stability [124], [125]. The exact sites of CaMKII phosphorylation on tubulin are unknown, but they are known to be on the C-terminus of both the α and β isoforms [124].

Following experimental refinement of these sites, or a guided guess (e.g. T312 is a strong candidate [175], [176]), a short peptide could be generated from the surrounding sequence of tubulin that would competitively inhibit the phosphorylation of endogenous tubulin by CaMKIIα. If p600 is acting through CaMKIIα and tubulin, the expression of this pseudo-substrate inhibitor ought to have the same effect as the inhibition of the p600/CaM interaction, namely predisposing neurons to Ca^{2+}-dependent death under ambient culture conditions. This intervention would furthermore need to be shown to be redundant with the expression of p600P, with each causing maximal death), and blocked by the inhibition of NMDA receptors.

The study of the effects of microtubule dynamics on Ca^{2+} signalling pathways has received very little attention in spite of its potential importance in regulating neuronal survival.
Whether it acts directly on microtubules or via CaMKIIα, p600 is an excellent candidate for a Ca\(^{2+}\)-dependent regulator of microtubule stability.

4.3 Conclusion

The data in this thesis show that when Ca\(^{2+}\) enters into a hippocampal neuron, a direct and atypical interaction between p600 and CaM prevents an immediate Ca\(^{2+}\)-induced dyshomeostasis, and ultimately prevents neuronal demise. Although excessive cytosolic Ca\(^{2+}\) normally bodes ill for hippocampal neurons, p600 is in a unique position to use an elevation of cytosolic Ca\(^{2+}\) to instead promote survival. Many acute (e.g. stroke and ischemia) and chronic pathologies (e.g. Alzheimer’s disease) involve Ca\(^{2+}\) overload (reviewed in [62], [177]–[179]. A loss of function of p600 could be central to the mechanisms of these pathologies. Conversely, study of the function of p600 in normal cells and tissues could further reveal how they handle Ca\(^{2+}\) overload without dying. Studying the role of p600 as a Ca\(^{2+}\) signalling protein in neurons \textit{in vivo} and in disease models will therefore be an important step not only in understanding p600, but also in understanding how neurons handle Ca\(^{2+}\) signals.
References


