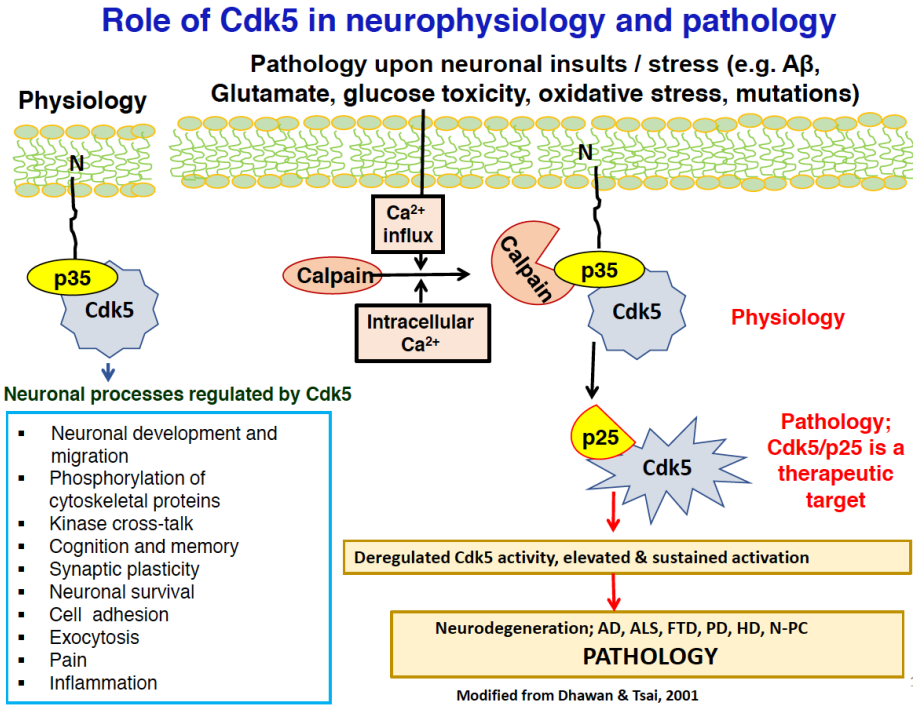

Figure 1: Regulation and deregulation of Cdk5 in physiology and pathology

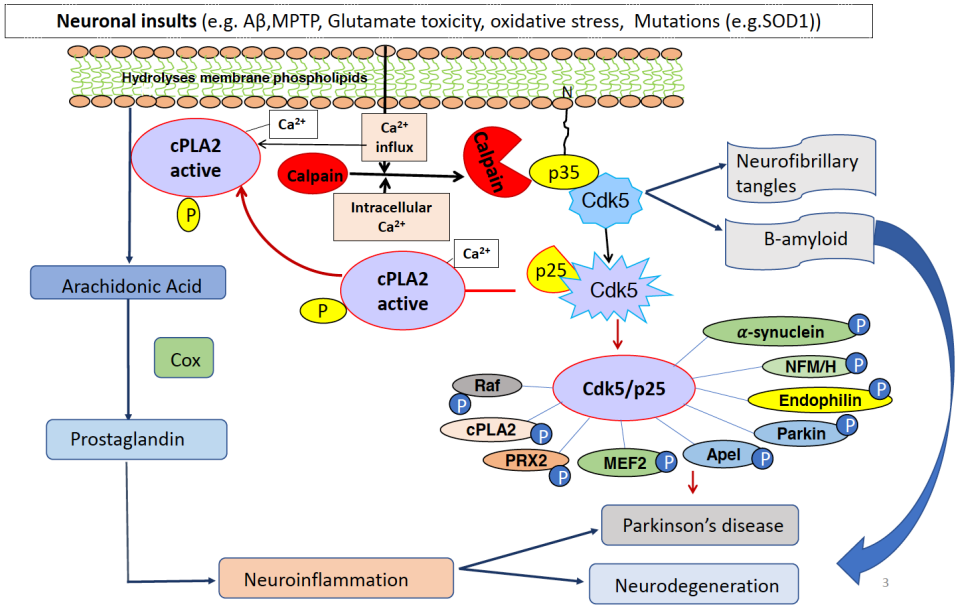

Figure 2: Signal transduction processes regulating neurodegeneration.

failing mitochondria, synaptic degradation, plaques and tangles [16]. A mitochondrial cascade hypothesis cites aging defects (mutations and stress) in mitochondrial metabolism as initiating events leading to defective mitochondria, oxidative stress, kinase activation, inflammation, DNA damage and the cognitive decline. Alternatively, evidence for the upstream role of hyperactive Cdk5/p25 has been shown in a number of different studies of cultured cells and model mice and has led to a hypothesis that continues to be tested (Figure 2).

The model assumes that neuronal insults and aging-induced upregulation of neuronal p25 is one of several etiological events triggering neurodegeneration and acts as age related mutations itself. The model proposes that this event leads to misfolding of proteins, oxidative defects in mitochondrial dysbioenergetics, inflammation, synaptic loss, plaques, tangles neuronal death, and behavioral decline [12]. The initiating event is a stress-induced upregulation of p25 by calpain cleavage of p35 into the P10 and p25 fragments [12]. P25

forms a more stable, deregulated cytoplasmic Cdk5/p25 hyperactive complex [12,17,18]. This toxic complex that contributes to metabolic events leading to neurodegeneration. In addition to cytoskeletal protein hyperphosphorylation, Cdk5/p25 hyperphosphorylation of other enzymes such as mitochondrial and other metabolic enzymes activities also affected and to enhance toxic effects. Perhaps the best example of p25-induced toxicity and progression of the AD phenotype is the p25 transgenic model mouse (p25Tg) [17,19,20] revealed all the AD phenotypes. We used bitransgenic (CK-p25Tg) mice (males and females) in which forebrain CK-p25 expression was tightly regulated by the tet on/off system facilitated by the CamKII (CK) promoter [17]. The tet-off system was induced by dietary alteration in doxycycline-supplemented food in which CK-p25 expression was suppressed in the presence of doxycycline. Calmodulin-dependent protein kinase II (CaMKII/CK) is a promoter used to activate the expression of the CK-p25 gene in cortical and hippocampal region of transgenic mice (p25 Tg). It was shown that CK-p25Tg mice develop significant neuronal loss in the cortex and hippocampus and display neurodegeneration and pathological tau hyper phosphorylation when p25 expression is induced. These mice are identified as p25Tg (over expressing). High p25 expression and elevated hippocampal Cdk5 activity has been observed within 1 weeks after induction.

All mice were developed and raised in the presence of doxycycline (DOX 1 mg/g in food). To induce CK-p25 expression, the mice were fed a normal diet, DOX-off. To inhibit p25 production, the mice were again fed a doxycycline diet. All experimental cohorts were treated under the same conditions and fed doxycycline diet for the same period of time Note that all animal procedures were performed in accordance with the NIH animal care committee's regulations.

Design and Synthesis of TFP5

TFP5 is a truncated portion of p35 (activator of Cdk5), which extends 24 aa residues in length (Lys254–Ala277) conjugated with an 11-aa modifying peptide derived from the trans-activator domain of TAT protein at the C terminus (to facilitate passage through the blood-brain barrier), while FITC, fluorescein isothiocyanate (a green fluorescent tag) with linker GGG, was attached at the N terminus (to serve as a marker). A scrambled peptide (Scb) was used as a control to TFP5 (sequence shown below). Peptide 2.0 (Chantilly, VA, USA) commercially synthesized both TFP5 and Scb peptides which were used after dissolving both in saline or double distilled water.

Sequences used were as follows:

TFP5,
FITCGGGKEAFWDRCLSVINLMSSKMLQINAYARAARRAARR

Scb peptide,
FITCGGGGGGFWDRCLSGKGKMSSKGGGINAYARAARRAARR

Intraperitoneal (I.p.) injection paradigm

Five cohorts of mice were used: vehicle-injected WT, CK-p25Tg+DOX, CK-p25Tg-DOX, TFP5-injected CK-p25Tg-DOX and Scb-injected CK-p25Tg-DOX. After 12 weeks, the 3 mutant cohorts were taken off DOX to activate the expression of CK-p25. All five cohorts were age-matched and WT, CK-p25Tg+DOX, CK-p25Tg-DOX was treated with i.p. injection of vehicle, while another group of -DOX mutant mice were injected with 40 mg/kg/d TFP5. As an additional control, a cohort of –DOX mutants was injected with 40 mg/kg/d Scb peptide. All five cohorts were injected 3 days/week on week's 13-17 for a total of 18 injections. All the mice were subjected to behavior analysis on week 18 and the mice were euthanized on week 19 when brain tissue was harvested for biochemical analysis. Based on the observation that injection of 3000 mg/kg/day of TFP5 into wild type mice in 5XFAD studies was not toxic, had no effect on body weight, behavior, appearance and longevity [21].

Conception, development and post-natal growth (at least to 12 days) are normal in the presence of doxycycline whereas removal of doxycycline induced over-expression of cortical p25, increased Cdk5 activity, neuronal loss, and progression to an AD-like phenotype with Ab plaques, hyper-phosphorylated tau tangles and inflammation [17,19]. It should be noted, in this transgenic, up regulation of p25 and hyperactivity of Cdk5 initiate inflammation via phosphorylation and activation of neuronal phospholipase 2A followed by astroglyosis and microgliosis which precede Abeta accumulation and tau phosphorylation [19]. In fact the situation has become even more confounding with the mixed results from other p25 mouse transgenic initiated at fertilization [22,23]. Robust p25 expression and Cdk5 activity at 4-5 months correlated with tau phosphorylation, axonopathy, neurodegeneration and severe motor defects but no evidence of other AD phenotypes. Moreover, later studies of the doxycyclin-regulated p25tg only added to the confusion; two roles for p25 were revealed, a low-dose, positive physiological role in memory formation, while a higher sustained p25 elevation induced neurodegeneration and AD-like phenotypes [24]. In our later examination of this transgenic, it is the latter response on which we focus.

p35-Derived Peptides as Therapeutic Candidates for Neurodegenerative Disorders

Clearly, Cdk5/p25 is a potential therapeutic target for neurodegeneration [25-27]. Although roscovitine and related compounds have been proposed and evaluated, their effects are non-specific as they bind the common ATP site shared by other Cdks and most other kinases. Our lab has taken a different approach based on a study of truncated fragments of the p35 regulator [28]. Two peptides were identified, CIP (126 a.a) and a smaller peptide p5 (24 a.a.), derived from the p25 domain of the parent sequence, exhibited vigorous inhibition of Cdk5/p35 and Cdk5/p25 activities in test-tube experiments [29-31]. Surprisingly, however, in cultured cortical neurons, the peptides inhibited only the Cdk5/p25 complex and spared the Cdk5/p35 kinase which retained most of its activity [31-33]. Here,E18 cortical neurons stressed by toxic Abeta display an AD phenotype, hyperactive Cdk5/p25, hyperphosphorylated tau and neurofilaments, Abeta accumulation and apoptosis. These effects are reduced when cells are incubated in different concentrations of p5 or CIP [11,29,32]. The specificity of peptide inhibition was dramatic; whereas Cdk5/p25 activity was inhibited; Cdk5/p35 activity was unaffected as were the activities of cyclin dependent kinases [32].

Wherein does this specificity reside? We assume that the inhibitor peptides compete with the physiological regulators for the catalytic site on the kinase, as suggested by computer modeling experiments and in the test-tube, using purified enzyme complexes, inhibition by peptides is effective and comparable for both complexes. The situation in cells, with numerous interacting proteins, differs fundamentally, however. It has been demonstrated that p35, with its p10 N-terminal "domain" interacts with other proteins such as microtubules, actin, munc18 and others forming a multimeric complex [6,7,31,33,34]. We suggest that binding of macromolecules to the p10 domain favors a p35 conformational change such that it competes successfully for the Cdk5 catalytic site and sustains activity. P25, without the p10 domain,

fails to compete successfully and is displaced by p5 which inhibits the kinase. An initial test of the hypothesis involved the incubation *in vitro* of microtubules to both Cdk5/p35 and Cdk5/p25 complexes followed by the addition of p5. Only the Cdk/p25 activity was inhibited [33]. A more extensive test of the role of the p10 domain in cultured neurons was carried out with Munc 18 a substrate of Cdk5 at the synapse [35,36]. Here, too, the activity of the Cdk5/p35 complex was spared in the presence of Munc 18 [31]. A key control was the observation that cortical neurons transfected with p67 siRNA exhibited inhibition of Cdk5/p35 in the presence of p5. Finally, the role of the p10 domain was confirmed in a pull-down experiment with GST-p10 which exhibited binding of Cdk5, p35 and p67 [31].

Therapeutic Effects of Peptides in Model Mice Showing Neurodegenerative Disorders

The real test of the hypothesis relies on studies of the effect of peptide treatment on those phenotypes in model mice resembling human disorders such as AD, ALS, PD and HD. A large, diverse selection of model mice has been engineered for each neurodegenerative disorder [37,38]. Most mouse models are prepared by introducing mutant human genes responsible for specific phenotypes. In AD, for example, transgenic bearing several known mutations of the APP pathway have been constructed which show a progression to AD resembling the phenotype in humans [38]. For the most part, most mouse models do mimic pathologies characterizing specific disorders. Behavioral criteria (memory, cognition, etc.), however, are difficult to duplicate in mice for many obvious reasons [37-39]. This raises the question as to whether such studies provide insight as to the nature and mechanisms of the human syndromes and may explain why clinical trials based on animal studies have been disappointing. Answers have been sought by comparisons of transcriptomes of brains from human patients and mimetic mouse models. Unfortunately, these reveal profound differences in the "up and down" expression of a wide range of genetic sets [40]. In general, mice are different from humans. Significantly, the authors report that mouse and human aging transcriptomes are more similar which suggests that overexpression of human gene mutations in transgenic mice distorts what is fundamentally natural aging. Nevertheless, the authors urge us to continue ways to improve the animal models.

Our approach is to carry on with tests of the hyperactive C

Cdk5/p25 hypothesis. We do so based on evidence of upregulated, deregulated Cdk5/p25 expression in AD brains [41], Cdk5/p35 activity (immunoprecipitated) associated with Lewy bodies in brains of Parkinson's and ALS patients [42-44] and in our own work showing upregulation of p25 in brain and spinal cord of five ALS patients with matched controls (unpublished data). Results of our studies of the therapeutic effects of the two peptides in mouse models of AD, PD and ALS are consistent with the proposed hypothesis.

Proof of Concept; the CK-Tgp25 Transgenic

As previously indicated, the p25Tg transgenic mouse, when induced by doxycycline removal, exhibits a significant upregulation of p25 and Cdk5 activity. In the earlier study most data were reported after 5-12 weeks of induction and showed upregulation of Cdk5/p25 activity correlated with tau phosphorylated aggregates, a neurofibrillary pathology [17]. A later study recorded earlier time points with Cdk5 activation evident after one week induction [19]. Virtually at the same time, inflammatory signs were seen with an uptick of GFAP at one week, cytokines and chemokines of microgliosis and phosphor-tau after 4 weeks induction, followed by Abeta, most evident at 8 weeks post induction. To study the therapeutic potential of the larger CIP peptide, mutants with p25Tg AD-like phenotype were crossed with normally appearing CIP double transgenics (producing TetraTg-CIP mice controlled by a CAMK2a promoter) over expressing p25 in a background of CIP inhibitor peptide overexpression in the brain [45]. Doxycycline removal for 1 weeks initiated overexpression of p25 and Cdk5 hyper activation in forebrains of p25Tg mice which were diminished in the TetraTg CIP x p25 Tg-expressing mice as was inflammation, tau phosphorylation and amyloid deposition; AD pathology was significantly reduced as were neuronal cell loss and neurocognitive defects. This is the first successful therapeutic targeting of Cdk5/p25 hyperactivity *in vivo* while sparing effects on Cdk5/p35 activity.

Analysis of the therapeutic effect of the smaller p5 peptide in the same p25Tg model mouse required a different approach. Here, the p5 peptide, modified for penetration of the blood-brain barrier as TFP5 was injected intraperitoneally [21,46].

Control and experimental animals were maintained on doxycycline for 12 weeks, then doxycycline was removed and a seriesof TFP5 injections (weeks 13 to 17) were given to p25Tg animals while controls received an identical series with scrambled peptide at the same concentration. Untreated animals showed the induced increase in p25 overexpression and Cdk5 activity whereas the TFP5 treated cohorts exhibited a 40% reduction in activity. They also showed reduced tau and neurofilament (NFM/H) phosphorylation, reduced inflammation and amyloid beta expression accompanied by improved behavioral function. Significantly there was an improvement in LTD expression, a sign that synaptic activity had been restored [46]. These studies suggest that neurodegenerative disorders expressing deregulated Cdk5/p25 may be therapeutically targeted with an appropriately designed inhibitor peptide derived from p35, the endogenous regulator in the brain.

The 5XFAD AD Model Mouse; the Effect of P5 Therapy

A different mouse model, familial 5XFAD, is a transgenic expressing human APP and PSI mutant genes (a total of five mutations). It overexpresses Abeta and amyloid plaques and exhibits significant defects in spatial memory and behavior [47]. Tau hyper phosphorylation and tangles are also evident as well as Cdk5/p25 hyperactivity. There is evidence that Abeta is toxic and induces tau hyper phosphorylation via activation of Cdk5/p25 in cortical neurons [29,33]. Moreover, intracerebroventricular injection of A beta in a mouse model hyper activates Cdk5/p25; it appears that Cdk5/p25 activation and Abeta are part of a circular feedback loop. For example, overexpression of p25 increases Cdk5-induced BACE1 transcription and the abnormal processing of APP [47,48]. Evidence from the effect of p5 peptide on the p5 overexpressing p25Tg transgenic does show the linkage between these metabolic pathways. Does it exhibit the same effect in the amyloid overexpressing model?

An injection protocol with TFP5 was established for this transgenic [21]. Control and experimental animals (6 months to 12 months of age) were injected intraperitoneally with and without TFP5 for three consecutive days (40 mg/kg) followed by a day of behavioral tests and sacrifice to dissect brains for biochemical and immunocytochemical analyses. TFP5 was shown to penetrate the blood-brain barrier; fluorescence was seen in cortex, hippocampus and cerebellum (as well as other organs) after four days. Significantly, after four days the hyper activation of Cdk5 was reduced in TFP5 treated animals to normal WT values; scrambled peptide controls had no effect. Coupled to

these changes were significant improvements in behavioral tests (e.g. Y maze) as well as reductions in inflammation, hyper phosphorylation of neurofilaments and the deposition of amyloid plaques [21].

Neuronal apoptosis was also decreased by 37% in the TFP5-treated mice. Here, too, the AD phenotype in this mouse model was successfully reduced only seven days after the last treatment, without affecting the endogenous Cdk5/p35 kinase activity. Moreover, by targeting the hyperactive Cdk5/p25 kinase, the Abeta phenotype is affected, consistent with the view that kinase activity and APP processing are linked, perhaps because Cdk5 phosphorylates the thr668 site on APP, a step in Abeta processing [48-50]. Differences in phosphorylation patterns at this site between Cdk5/p35 and Cdk5/p25 were reported, the former phosphorylating both mature and immature APP while the latter only increased phosphorylation of the immature form [47]. Hence, by targeting Cdk5 we show specific effects of the peptide inhibitor on other pathological pathways underlying neurodegeneration.

Parkinson's Disease: Effect of P5 Peptide on the MPTP Mouse Model

Parkinson's disease is one of the consequences of aging and is increasing globally as the world population ages. This disorder, specific to substantia nigra cells, is also characterized by aggregate accumulation, synuclein-containing Lewy bodies that lead to neuronal death. Synuclein aggregates reflect dysregulation of the autophagy pathways that modulate the degradation of misfolded and abnormal proteins [51]. It is noteworthy that these aggregates also contain Cdk5 [42,43,52]. Moreover, post mortem studies of PD brains show evidence of calpain-induced Cdk5/p25 activation [53]. Mutations in a human ubiquitin-protein ligase, Parkin, also contribute to the AD phenotype [54,55]. Parkin, hyper phosphorylated by Cdk5, become dysfunctional and leads to the accumulation of damaged proteins [56]. As in AD, Cdk5 plays a key role in etiology of the PD phenotype.

Several mouse models of Parkinsons have been produced, e.g. transgenics overexpressing a dominant alpha synuclein mutant), but a more common model develops after treatment with a drug, 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP) which specifically destroys dopaminergic neurons in the substantia nigra of the brain leading to neuronal death [57]. Neuron loss is correlated with the overexpression of Cdk5 which may be responsible for mitochondrial dysfunction and oxidative stress, coupled to deregulated protein folding and autophagy [58].

We have used this PD model to study the effect of TFP5 and TP5 peptides on the expression of the abnormal Parkinson's phenotype [59,60]. Induction of hyperactive Cdk5/p25 was confirmed in mesencephalic cultures treated with MPTP, which was significantly reduced when cells were pretreated with TFP5, as was cell survival. Inflammation of primary mesencephalic cells was also ameliorated by peptide pretreatment, before incubation in MPTP. More to the point, potential therapeutic effects *in vivo* were tested in a four-dose MPTP model mouse [61]. Animals were injected intraperitoneally with TP5/TFP5 at a high dose (80 mg/kg) for 9 days; on day 2, however, they had received the four doses of MPTP. Here, too, Cdk5/p25 activity was reduced in the substancia nigral cells, as was inflammation, dopamine levels and cell death. No protection was afforded by pretreatment with the control scrambled peptide.

It is not clear that Cdk5/p25 activation is the upstream trigger for induction of the MPTP phenotype; its mechanism of action is confounded by the fact that cross talk between Cdk5 and other kinases is a common feature in the brain. Moreover, the numerous substrate targets of Cdk5 may be affected [62] including some implicated in MPTP pathology involving mitochondrial function such as cytochrome c release, caspase-3 activation and reduction of the anti-oxidant Prx2. These aspects of mitochondrial activity were protected after TFP5 treatment.

It should be pointed out that transcriptome analyses of PD mouse brains as contrasted with human PD transcriptomes differ significantly, at least with respect to the more than 250 unregulated genes in human brains that are not matched in the mouse models [40]. Downregulated genes, however, exhibit a greater match. These discrepancies between mouse and human characterize the transcriptome data for other neurodegenerative disorders [40], which may account for the fact that primate models for PD have been introduced [40]. Future tests of the peptides in these primate models of PD might bring us closer to clinical trials in human patients.

Brain Ischemia; Cdk5 Activation and Regulation by P5 Peptides

Stroke, a leading cause of death world-wide, is responsible for short and long term cognitive impairment, i.e., declines in memory, learning and executive functions. There are reports that Cdk5 activity is upregulated in human stroke calpain upregulation and hyper activation of Cdk5/p25 have been identified in animal models of ischemia and may be responsible for downstream pathologies associated with neuronal death [63-65]. Accordingly, hyper activated Cdk5/p25 has been suggested as a target for therapeutic intervention after a stroke [66]. Several approaches such as roscovitine-like inhibitors [67] or Cdk5 silencing by Cdk5 RNAi induced neuroprotection; in the case of the latter, treatment resulted in reversal of learning defects and memory after one and four months post ischemic induction in rats [68,69]. The treatment prevented neuronal loss, inflammation, tau pathology as well as a behavioral deficit, including hippocampal long term potentiation. Upregulation of BDNF in the hippocampus may have been a contributing factor.

The pattern of Cdk5/p25 activation after an ischemic episode resembles that of other neurodegenerative disorders and invites the application of the p5 peptide therapeutic strategy so successful in AD and PD [70]. Using a hypoxia/ischemic insult in neonatal rats on post-natal day 7, brains from experimental (intraperitoneal injected p5-TAT (TP5) treated to facilitate crossing the blood-brain barrier) and sham controls (untreated) were compared as to levels of p25, p35 and Cdk5 activity at different time points post ischemia. P35 decreased after the insult whereas p25 increased robustly as did Cdk5/p25 activity. The p5-TAT treatment, however, had no effect on the levels of the regulators, but hyperactive Cdk5/p25 was diminished as seen in the reduction in levels of phospho-tau and phosphorylation of the glucocorticoid receptor [70]. After seven daily injections of p5-TAT post insult, behavioral studies also pointed to a successful improvement. Again, we see that in those neuronal disorders marked by a significant up-tick of calpain activity, p25 upregulation and hyperactive Cdk5/p25 phosphorylation of substrates such as tau, treatment with an inhibitory peptide derived from the p35 kinase regulator successfully attenuates pathology, neuronal death and compromised behavior.

Regulation of Insulin Secretion in Pancreas; Cdk5 Activity and Peptide Inhibition

Cdk5 kinase activity is not restricted to the nervous system; it plays a role in diverse cell types including muscle, pancreas and even

cancers, among others [71]. It is involved in transcription regulation, muscle differentiation, cell migration and adhesion and in insulin regulation of glucose uptake. Studies of its role in type 2 diabetes, have led to conflicting results. Although Cdk5 and its activators, p35 and p39 are expressed in pancreatic islets and beta cell lines, some reports claim its activation promotes insulin secretion [72,73], while others report that knockout of p35 and inhibition of Cdk5 activity promotes insulin secretion [74,75]. We have shown that duration of glucose toxicity is a key factor in regulation; short term (2 h) resulted in a modest increase in Cdk5 activity and insulin secretion whereas long term exposure results in significantly enhanced Cdk5 activity and a decline in insulin secretion [33]. Overexpression of p35 in Min6 cells plus high glucose toxicity is a stress signal that induces p25 expression and Cdk5 hyperactivation. The end result is a significant inhibition of insulin secretion. This pattern of Cdk5 deregulation resembles that seen in some neuronal disorders and suggests that inhibition of Cdk5 may promote insulin secretion under these conditions, which it does when using roscovitine or dnCdk5 transfection [33]. These non-specific results, however, fail to distinguish between inhibition of Cdk5/p35 or Cdk5/p25 activities (or both. Subsequently, we have shown that CIP, the large peptide that specifically inhibits Cdk5/p25 does rescue insulin secretion at high glucose [76,77], as does the smaller peptide TFP5 [78].

Results showing peptide inhibition of Cdk5 deregulation in non-neuronal cells invites speculation that many diverse organs and tissues in which Cdk5 plays a key role, that the peptides may also affect those disorders marked by hyperactive Cdk5 induced by toxic overexpression of p25.

Conclusion

The protein kinase Cdk5 is ubiquitous, found in most mammalian cells and tissues, where, because of its wide range of targeted substrates, is involved in key signaling pathways and kinase cross-talk. It is tightly regulated physiologically by non-cyclin activators, p35, p67 and p39 and as a Cdk5/p35 complex, is essential in the development of the nervous system, synaptogenesis, synaptic function and neuronal survival.

Under neuronal stress (aging, mutations, environmental insults) the kinase is deregulated; increased calcium flux evokes activation of the proteinase calpain, cleavage of p35 (and or p39) into a p10 myristoylated N-terminal fragment and p25, a hyperactive regulator which stably binds Cdk5, hyperactivates it and induces cellular pathology (protein aggregates) in several neuronal disorders. Similarly deregulated in other cells and tissues it provokes cell specific pathologies (e.g. insulin secretion). Accordingly, the Cdk5/p25 complex has been identified as a therapeutic target.

We have produced two small peptides, (CIP, 126 and P5, 24 aa) truncated fragments of p35, which specifically inhibit the Cdk5/p25 and Cdk5/p35 complexes *in vitro* but in cultured cells and *in vivo* in model mice, CIP and p5 specifically inhibits the abnormal Cdk5/p25 without affecting activities of Cdk5/p35 nor of related Cdks, the cell cycle kinases. Treatment with the peptides successfully reduces pathologies and behavioral defects in mouse models of AD, PD, ischemia and type 2 diabetes. Following are some of the novelties of CIP and P5 peptides; The novelty of these peptide is absence of toxicity (>3000 mgm/kg in mice) and have higher affinity with Cdk5 compared to ATP. These peptides are very stable at room temperature.

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