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Estrogen Regulation of Dkk1 and Wnt/β-Catenin Signaling in Neurodegenerative Disease

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Abstract

17β-estradiol (E2 or estrogen) is an endogenous steroid hormone that is well known to exert neuroprotection. Along these lines, one mechanism through which E2 protects the hippocampus from cerebral ischemia is by preventing the post-ischemic elevation of Dkk1, a neurodegenerative factor that serves as an antagonist of the canonical Wnt signaling pathway, and simultaneously inducing pro-survival Wnt/β-Catenin signaling in hippocampal neurons. Intriguingly, while expression of Dkk1 is required for proper neural development, overexpression of Dkk1 is characteristic of many neurodegenerative diseases, such as stroke, Alzheimer's disease, Parkinson's disease, and temporal lobe epilepsy. In this review, we will briefly summarize the canonical Wnt signaling pathway, highlight the current literature linking alterations of Dkk1 and Wnt/β-Catenin signaling with neurological disease, and discuss E2's role in maintaining the delicate balance of Dkk1 and Wnt/β-Catenin signaling in the adult brain. Finally, we will consider the implications of long-term E2 deprivation and hormone therapy on this crucial neural pathway.

Keywords

Brain; Dkk1; Estrogen; Neurodegenerative Disease; Wnt Signaling

1. Introduction – Estrogen as a Neuroprotective Agent

17β-estradiol (E2 or estrogen) is an endogenous steroid hormone produced in the ovaries and in the brain. In addition to its well-known roles in reproduction, bone homeostasis, and metabolic functions, E2 also serves as a neuroprotective agent. In support of this, premenopausal women, who have high circulating E2 levels, are relatively protected from neurodegenerative diseases, such as stroke, when compared to men (Brann et al., 2007; Murphy et al., 2004; Niewada et al., 2005; Roquer et al., 2003; Scott et al., 2012). In contrast, this pattern is reversed for postmenopausal women, who demonstrate dramatically reduced circulating E2 levels and actually have worse morbidity and mortality following a stroke than age-matched men (Appelros et al., 2009; Niewada et al., 2005; Persky et al., 2010; Roquer et al., 2003). Importantly, animal models of stroke corroborate the phenomenon of E2 neuroprotection observed in humans. In fact, male rodents demonstrate larger cortical infarcts than their female counterparts after cerebral ischemia (Alkayed et al., 1998; Roof and Hall, 2000), and both estrogen receptor antagonists and aromatase

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inhibitors, which block endogenous production of E2, enhance stroke damage in rodent models of cerebral ischemia (McCullough et al., 2003; Sawada et al., 2000). Intriguingly, since exogenous estrogens also afford neuroprotection in the cerebral cortex and hippocampus in animal models of cerebral ischemia (Brann et al., 2007; Gibson et al., 2006; Scott et al., 2012; Simpkins et al., 2012a; Simpkins et al., 2012b), E2 has the potential to serve as an acute treatment for stroke and/or as a preventative therapeutic to preserve optimal neuronal functioning in postmenopausal women. Along these lines, it is no surprise that the mechanisms of E2 neuroprotection and the feasibility of postmenopausal hormone therapy are areas of intense study. One mechanism proposed to contribute to E2 neuroprotection is E2's attenuation of ischemic elevation of the neurodegenerative Wnt antagonist dickkopf-1 (Dkk1) and simultaneous activation of the pro-survival Wnt/β-Catenin pathway (Zhang et al., 2008). Thus, below we review canonical Wnt/β-catenin signaling and its regulation by the Wnt antagonist Dkk1, as well as the role of Dkk1 in neurodegenerative disorders and its regulation by estrogen.

2. Canonical Wnt/β-Catenin Signaling

Wnt is a secreted glycoprotein whose gene was separately discovered in mouse mammary tumors (int-1) and in *Drosophila melanogaster* (wingless), but due to sequence homology, both int-1 and wingless were determined to encode the same proto-oncogene, which was dubbed Wnt (McMahon and Moon, 1989a; McMahon and Moon, 1989b; Thomas and Capecchi, 1990). The canonical Wnt signaling pathway has since been deemed critical for several embryonic events, including cell proliferation, cell polarity, and determination of cell fate (Logan and Nusse, 2004; MacDonald et al., 2009). Canonical Wnt/β-Catenin signaling has also been implicated in development of the limbs (Grotewold and Ruther, 2002; Mukhopadhyay et al., 2001), neural tube (De Marco et al., 2012; Roelink and Nusse, 1991), forebrain (Mukhopadhyay et al., 2001), midbrain and cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and in the maintenance of neurotransmission and synaptic plasticity (Ataman et al., 2008; Avila et al., 2010; Budnik and Salinas, 2011; Jensen et al., 2012; Speese and Budnik, 2007). In light of this knowledge, it is not surprising that, in addition to tumorigenesis, mutations that enhance Wnt signaling in humans have been linked to neurological disorders, such as autism, schizophrenia, and bipolar disorder (De Ferrari and Moon, 2006).

Wnt initiates an intracellular signaling cascade when it binds to its cognate membrane receptor, frizzled (Fz), and its co-receptor, low density lipoprotein-related protein 5/6 (LRP5/6) (Logan and Nusse, 2004; MacDonald et al., 2009) (**See** Figure 1 **for Summary**). The canonical Wnt signaling cascade, which ultimately determines intracellular levels of the transcriptional activator β-Catenin, begins with recruitment of disheveled (Dvl) to Fz and dual phosphorylation of LRP5/6 by glycogen synthase kinase 3β (GSK3β) and casein kinase I (CKI) (MacDonald et al., 2009). It is important to note that Wnt is also capable of signaling through non-canonical signaling pathways, which are independent of β-Catenin, but these are beyond the scope of this review. As such, the reader is referred to these excellent reviews on the subject (Clark et al., 2012; Komiya and Habas, 2008; Seifert and Mlodzik, 2007; Wang and Nathans, 2007). Intriguingly, GSK3β and CKI are both components of the Axin-adenomatous polyposis coli (APC) complex, which is devoted to the phosphorylation of the transcriptional activator β-Catenin (Logan and Nusse, 2004; MacDonald et al., 2009). As such, in the absence of Wnt ligand, the Axin-APC complex continually phosphorylates cytosolic β-Catenin, priming it for ubiquitination by the E3 ubiquitin ligase Beta-Transducing repeat-Containing Protein (β-TrCP) and subsequent proteasomal degradation in order to prevent expression of Wnt target genes. However, once Wnt signaling is initiated, GSK3β and CKI are recruited away from the Axin-APC complex to phosphorylate the co-receptor LRP5/6, and once doubly phosphorylated, LRP5/6

becomes a docking site for Axin (MacDonald et al., 2009). These events lead to the temporary disassembly of the Axin-APC complex and the subsequent stabilization of cytosolic β-Catenin, which remains untagged by either phosphate or ubiquitin (Logan and Nusse, 2004; MacDonald et al., 2009). Thus, the canonical Wnt signaling cascade ultimately ends in the elevation, stabilization, and nuclear translocation of cytosolic β-Catenin. Once inside the nucleus, β-Catenin, as a transcriptional activator, is able to convert the T-cell Factor/Lymphoid Enhancing Factor (TCF/LEF) transcriptional repression machinery into an active, mRNA transcription complex, which then promotes the expression of Wnt target genes, characterized by the presence of a consensus sequence called a Wnt response element (WRE) in their promoter regions (Logan and Nusse, 2004; MacDonald et al., 2009).

3. Dkk1

Several endogenous ligands, such as Shisa, Wnt inhibitory factor-1 (WIF-1), the secreted Frizzled related proteins (sFRPs), the WISE/SOST family, and the Dickkopf (Dkk) family, can serve as antagonists of canonical Wnt signaling (MacDonald et al., 2009). Arguably, the most important Wnt signaling antagonist is the prototypical Dkk family member Dkk1, which antagonizes Wnt signaling by binding to the LRP5/6 co-receptor and preventing Wnt from forming a signaling complex with Fz and LRP5/6 (Bafico et al., 2001; Fedi et al., 1999; Mao et al., 2001; Semenov et al., 2001; Wu et al., 2000). Similar to Wnt, Dkk1 expression is critical for neurodevelopment during the embryonic period. In fact, Dkk1 is known as a “head-inducer,” as its presence and antagonism of Wnt signaling is required for structures anterior of the midbrain to form (Glinka et al., 1998; Kazanskaya et al., 2000; Mukhopadhyay et al., 2001; Semenov et al., 2001). Importantly, Dkk1 is also responsible for orchestrating the apoptosis necessary for proper limb development (Grotewold and Ruther, 2002; Mukhopadhyay et al., 2001). Along these lines, Dkk1 null mice are not viable, with embryos lacking structures anterior of the midbrain and demonstrating limb polysyndactyly (Mukhopadhyay et al., 2001). Furthermore, doubleridge mice, which have hypomorphorphic expression of Dkk1, are viable, but they display hemivertebral fusions and polysyndactyly of the forelimbs, a phenotype that can be ameliorated by reducing the expression of LRP5/6 (MacDonald et al., 2004).

3.1. Dkk1 and Neurodegenerative Disease

While transgenic mouse models have overwhelmingly demonstrated that Dkk1 expression is crucial during neurodevelopment, elevation of Dkk1 later in life can be detrimental. In fact, many studies have linked elevated Dkk1 expression in the adult brain to neurodegenerative diseases, such as stroke, Alzheimer’s disease, Parkinson’s disease, and temporal lobe epilepsy (**See** Table 1 **for Summary**). Excitotoxicity has relevance to neurodegenerative disease because stroke leads to neuronal cell death, in part, due to excess release of the excitatory neurotransmitter glutamate, which subsequently leads to NMDA receptor activation and intracellular calcium overload (Choi, 1994a; Choi, 1994b; Zipfel et al., 2000). *In vitro* studies demonstrated that Dkk1 is induced in cultured cortical neurons following an excitotoxic pulse of NMDA and is also capable of potentiating NMDA neurotoxicity in a dose-dependent manner (Cappuccio et al., 2005). Further work confirmed that Dkk1 is, in fact, able to inhibit canonical Wnt signaling and initiate cell death in cultured cortical neurons, which was associated with loss of Bcl-2, induction of Bax, and hyperphosphorylation of the microtubule associated protein tau (Scali et al., 2006). Importantly, the same studies confirmed that these observations are relevant to ischemic insults *in vivo*, as hippocampal Dkk1 was induced following global cerebral ischemia in both gerbils and rats, and stereotaxic injection of recombinant Dkk1 into either the hippocampal CA1 region or nucleus basalis magnocellularis was sufficient to cause neuronal cell death (Cappuccio et al., 2005; Scali et al., 2006). Intriguingly, intracerebroventricular

injection of Dkk1 anti-sense oligonucleotides attenuated the ischemia-induced cell death observed in gerbils, and intraperitoneal administration of lithium chloride, which rescues canonical Wnt signaling by inhibiting GSK3β, also attenuated the ischemia-induced cell death observed in rats (Cappuccio et al., 2005).

A later study demonstrated that neural Dkk1 is also induced in animal models of focal cerebral ischemia (local endothelin-1 infusion and permanent middle cerebral artery occlusion [MCAO]) and reiterated that administration of lithium ions was neuroprotective in rodents (Mastroiacovo et al., 2009). The same authors also performed MCAO in doubleridge mice, which have reduced expression of Dkk1, and noted a significant reduction in cortical infarct volume (Mastroiacovo et al., 2009). As such, these studies demonstrate the importance of the Wnt antagonist Dkk1 in the pathophysiology of cerebral ischemia and suggest that Dkk1 antagonists and/or Wnt agonists may be effective treatments for stroke. Finally, a recent study associated elevated circulating Dkk1 levels with acute ischemic stroke (<24 hours) in humans. While there was no relationship between Dkk1 and stroke severity or outcome, the authors found that plasma levels of Dkk1 were significantly higher in patients presenting with acute ischemic stroke versus healthy controls or patients with clinically stable cerebrovascular disease (Seifert-Held et al., 2011). Intriguingly, this study is in agreement with findings by Kim et al., which suggested that Dkk1 was elevated in the plasma of patients with coronary atherosclerotic plaques, even if they demonstrated low Agatston calcium scores (Kim et al., 2011). Thus, in addition to being a plausible therapeutic target for stroke, the Wnt antagonist Dkk1 may also be a promising biomarker for cardiovascular and/or cerebrovascular disease.

Several studies have also implicated dysregulation of Dkk1 and Wnt/β-Catenin signaling in Alzheimer's disease (AD), both in familial/early-onset AD and in sporadic/late-onset AD [For review, see (De Ferrari and Moon, 2006) and (Boonen et al., 2009)]. In regard to Dkk1, Caricasole and colleagues noted that the beta-amyloid peptide induced expression of Dkk1, hyperphosphorylation of tau, and cell death in cultured cortical neurons (Caricasole et al., 2004). Furthermore, they showed that anti-sense knockdown of Dkk1 *in vitro* attenuated beta amyloid neurotoxicity and prevented the hyperphosphorylation of tau, which forms neurofibrillary tangles, one of the neuropathological hallmarks of AD (Caricasole et al., 2004). Along these lines, the same authors also observed enhanced Dkk1 expression in neurons from post-mortem human AD specimens, which consistently co-localized with neurofibrillary tangles of hyperphosphorylated tau (Caricasole et al., 2004), suggesting that Dkk1 may play an important role in human AD neuropathology. A subsequent study revealed that Dkk1 was also upregulated in transgenic mouse models of AD and fronto-temporal dementia (Rosi et al., 2010). In particular, Rosi et al. noted that Dkk1 was significantly increased in brain regions affected by the respective neurodegenerative disease and co-localized with neurons containing neurofibrillary tangles, similar to what is seen in humans (Rosi et al., 2010). Additionally, in the TgCRND8 mouse model of AD, Dkk1 was expressed in choline acetyltransferase-positive neurons of the basal forebrain, neurons thought to be primarily affected by AD, and in neurons adjacent to beta-amyloid deposits (Rosi et al., 2010). Recent work also demonstrated that acute treatment with oligomeric beta-amyloid enhanced Dkk1 expression and led to a loss of synapses, which occurs early in the pathophysiology of AD and may facilitate cognitive impairment (Purro et al., 2012). The authors further showed that brief exposure to Dkk1, through the inhibition of Wnt signaling, decreased the size of both presynaptic and postsynaptic terminals in mature neurons without affecting cell viability and disassembled synapses within hours by inducing the release of synaptic vesicles (Purro et al., 2012). Intriguingly, they also showed that antibodies capable of neutralizing Dkk1 suppressed the aforementioned synapse loss in mouse hippocampal slices (Purro et al., 2012). As such, these results support the idea that Dkk1 could be responsible for synaptic loss in the early stages of AD and further suggest that Dkk1 may

serve a feasible target for the treatment of AD. It is important to mention that while no conclusive evidence has been provided, Dkk1 was identified in two screens for late-onset AD susceptibility genes (Morgan et al., 2007; Morgan et al., 2008). Furthermore, reduced Wnt/β-Catenin signaling has already been associated with genetic susceptibility to late-onset AD. Along these lines, a common variant of the LRP6 co-receptor (Val-1062), which has reduced β-Catenin signaling *in vitro*, was shown to interact with apolipoprotein E-epsilon4 (APOE-ε4) carrier status to form a risk haplotype for AD (De Ferrari et al., 2007). These results are in agreement with several studies implicating reduced Wnt signaling and activation of GSK3β, a kinase downstream of Dkk1 that is known to phosphorylate the microtubule associated protein tau, with various neurodegenerative diseases [See (Lei et al., 2011) for Review] and AD, in particular (Forlenza et al., 2011; Rockenstein et al., 2007).

Intriguingly, Dkk1 has also been implicated in two different animal models of Parkinson's disease. L'Episcopo et al. demonstrated that reactive astrocytes may afford neuroprotection against 1-methyl-4-phenylpyridinium (MPP^+) neurotoxicity by upregulating Wnt1 in the ventral midbrain and striatum *in vivo* (L'Episcopo et al., 2011). Importantly, the same authors also noted that blocking canonical Wnt signaling with Dkk1 prevented astrocyte-induced neuroprotection *in vitro* (L'Episcopo et al., 2011). Furthermore, Dkk1 was found to be upregulated in rats after stereotaxic administration of the selective dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA), and administration of Dkk1 was found to potentiate the neurotoxicity of 6-OHDA in the substantia nigra *in vivo* (Dun et al., 2012). As such, these recent studies suggest that Dkk1 may play a role in the pathobiology of Parkinson's disease and warrant further study in the human condition. Finally, a single study linked Dkk1 expression and subsequent Wnt inhibition to neurodegeneration caused by temporal lobe epilepsy. Busceti et al. demonstrated that Dkk1 was elevated in rat olfactory and hippocampal neurons following systemic administration of kainate, a compound known to induce seizure activity (Busceti et al., 2007). Intriguingly, Dkk1 was only induced in rats that were characterized as "high responders" to kainate, displayed reduced levels of nuclear β-Catenin, and experienced neuronal cell death following seizures. Furthermore, the researchers showed that either knockdown of Dkk1 or pre-treatment with lithium ions was sufficient to reduce kainate-induced cell death (Busceti et al., 2007). Importantly, Dkk1 was also strongly expressed in brain biopsies from patients with mesial temporal lobe epilepsy and hippocampal sclerosis, further implicating Dkk1 in the neurodegenerative processes associated with this disorder (Busceti et al., 2007). Thus, as a whole, these studies suggest that elevations in the Wnt antagonist Dkk1 in adulthood and subsequent reductions in canonical Wnt signaling are associated with neurodegenerative disease.

4. Estrogen Regulation of Dkk1 and Wnt/β-Catenin Signaling

Intriguingly, E2, as an endogenous steroid hormone, is not only capable of preventing the neuronal damage associated with neurodegenerative diseases, but is also able to promote a favorable balance of Dkk1 and Wnt signaling in the brain. As mentioned earlier, our laboratory reported that one mechanism through which E2 prevents neuronal cell death from global cerebral ischemia (GCI) is by suppressing the post-ischemic elevation of Dkk1 and simultaneously facilitating pro-survival Wnt/β-Catenin signaling in the CA1 hippocampal region (Zhang et al., 2008). Using Western blotting, we showed that Dkk1 is significantly upregulated in ischemic animals at 24 and 48 hours following GCI, and we demonstrated that a low, Diestrus I dose of E2, given via subcutaneous osmotic mini-pump one week before induction of ischemia, was able to prevent this elevation at both of these post-reperfusion time points (Figure 2). Furthermore, we showed that Dkk1 expression co-localized with NeuN, a neuronal marker, suggesting that Dkk1 is primarily expressed in *neurons* 24 hours after GCI (Figure 2). In the same study, we also examined the status of canonical Wnt signaling in the CA1 hippocampal region after GCI and E2 treatment by

measuring protein levels of Wnt3, phospho-β-Catenin, nuclear β-Catenin, and the canonical Wnt signaling product Survivin. In addition to elevations of Wnt3 and nuclear β-Catenin, we observed that E2 treatment not only prevented GCI-related loss but also enhanced the neuronal expression of Survivin, a Wnt target gene that facilitates survival through preventing the cleavage of pro-apoptotic caspases, at 24 and 48 hours after GCI (Figure 3). Since these are the same time points that we noted significant E2 suppression of the Wnt antagonist Dkk1 following GCI, we concluded that E2 is able to maintain a favorable balance of Dkk1-Wnt/β-Catenin signaling in the hippocampus following GCI. Finally, we demonstrated that E2's post-ischemic suppression of Dkk1 elevation is required for its neuroprotective ability, as intracerebroventricular injection of Dkk1 peptides into E2-treated animals was sufficient to reverse E2 neuroprotection status (Figure 4).

While our lab demonstrated that pre-treatment with low-dose E2 increased expression of Wnt3 in CA1 hippocampal neurons 24 and 48 hours following GCI and led to increased expression of Survivin, a Wnt target gene, E2 regulation of β-Catenin-dependent transcription may also occur independently of canonical Wnt signaling. However, regardless of the mechanism involved, the evidence overwhelmingly suggests that E2 stabilizes β-Catenin via inhibition of GSK3β [See (Varea et al., 2010) for review]. In fact, E2 effectively increases the amount of inactive GSK3β in neuronal cells *in vitro* (Cardona-Gomez et al., 2004; Varea et al., 2009), and E2 treatment protects hippocampal slice cultures from kainate-induced neurotoxicity via rapid, ER-mediated phosphorylation and inactivation of GSK3β at Serine 9 (Goodenough et al., 2005). Cardona-Gomez et al. further demonstrated that acute E2 treatment increased the amount of inactivated GSK3β in the rat hippocampus, which subsequently prevented the hyperphosphorylation of tau (Cardona-Gomez et al., 2004). This is consistent with our previous study, which showed that E2 increased the amount of inactive GSK3β at 24 and 48 hours following GCI and led to the stabilization of β-Catenin and the prevention of tau hyperphosphorylation at these same time points (Zhang et al., 2008). Varea et al. further demonstrated that estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) both directly interact with GSK3β and β-Catenin *in vitro* and that E2, PPT (an ERα agonist), and DPN (an ERβ agonist) were all capable of inducing β-Catenin-mediated transcription through the TCF/LEF-1 family of transcription factors in primary cortical neurons and neuroblastoma cells (Varea et al., 2009). Importantly, they observed that the ER antagonist ICI 182780 essentially blocked E2's induction of TCF/LEF-1-mediated transcription, further suggesting that E2's stabilization of β-Catenin requires the estrogen receptor, and they noted that PPT was more effective than DPN, suggesting that while both ERα and ERβ can mediate E2's effect on β-Catenin-mediated transcription in the brain, E2 may be acting primarily through ERα to upregulate TCF/LEF-1-mediated transcription *in vitro* (Varea et al., 2009). Finally, two independent studies suggested that E2-induced β-Catenin-mediated transcription requires LEF-1 and activates a set of genes that is similar, but not identical to, that activated by canonical Wnt signaling (Varea et al., 2009; Wandosell et al., 2012). These observations further promote the idea that, in addition to regulating the canonical Wnt signaling pathway, E2 can act independently of the canonical Wnt signaling pathway to enhance β-Catenin-mediated transcription in neural cells.

Recently, it has been determined that hypothalamic β-Catenin expression naturally fluctuates across the estrous cycle in rats, with elevations of β-Catenin observed during proestrus and estrus, two days characterized by high, circulating levels of E2 (Barrera-Ocampo et al., 2012). Intriguingly, the peaks in hypothalamic β-Catenin expression were concomitant with increases of activated Akt and inactivated GSK3β, suggesting that these two events are critical for E2-induced stabilization of β-Catenin in the brain (Barrera-Ocampo et al., 2012). E2 is well known to activate the neuroprotective phosphatidylinositol 3 kinase (PI3K)-Akt signaling pathway, either by itself or in concert with IGF-1 signaling, leading to the

activation of the serine/threonine kinase Akt through phosphorylation at Serine 473 (Brann et al., 2007; Burgering and Coffer, 1995; Mendez et al., 2003; Singh, 2001; Varea et al., 2010). Importantly, activated Akt has also been shown to inhibit GSK3β via phosphorylation at Serine 9 (Cross et al., 1995), and Mendez et al. demonstrated that blockade of the PI3K-Akt pathway with wortmannin, a specific PI3K inhibitor, decreased the amount of cytosolic β-Catenin in neuronal cultures (Mendez and Garcia-Segura, 2006). In light of this knowledge, Varea et al. propose a novel, additional mechanism for E2's regulation of β-Catenin-mediated transcription, where E2 binds to membrane-bound ERα and rapidly activates the neuroprotective PI3K-Akt pathway, possibly through interaction with IGF-1 signaling components. A multi-molecular complex is then formed consisting of ERα, GSK3β, β-Catenin, and others (Varea et al., 2009; Varea et al., 2010). Once activated via phosphorylation at Serine 473, Akt then inactivates GSK3β, which leads to the subsequent stabilization and nuclear retention of cytosolic β-Catenin. Inside the nucleus, β-Catenin then interacts with the TCF/LEF-1 transcription machinery to promote the expression of target genes that are independent of Wnt (Varea et al., 2009; Varea et al., 2010; Wandosell et al., 2012). Thus, it is plausible that E2 may maintain the delicate balance of Dkk1 and Wnt/β-Catenin signaling in the adult brain in three different ways: 1) by suppressing neuronal expression of the neurodegenerative Wnt antagonist Dkk1 2) by enhancing Wnt3 expression and subsequently facilitating canonical Wnt/β-Catenin signaling in neurons, and 3) by promoting Wnt-independent, β-Catenin-mediated transcription through a membrane ER-initiated intracellular signaling cascade involving PI3K/Akt/GSK3β (Figure 5).

5. Future Directions – Long Term Estrogen Deprivation and Therapeutics

While it is apparent that elevations in Dkk1 and reductions in Wnt/β-Catenin signaling are associated with neurodegenerative disease and that E2 may prevent neurodegenerative disease through its favorable effect on neural Dkk1 and Wnt/β-Catenin signaling, many questions remain unanswered. Importantly, women who enter menopause prematurely (< 45 years of age) have increased risks for ischemic stroke, dementia, and mortality from neurological disorders, which can be ameliorated with timely replacement of E2 until the age of natural menopause (Rivera et al., 2009; Rocca et al., 2007; Rocca et al., 2011; Rocca et al., 2012; Shuster et al., 2010). This suggests that dramatic and prolonged loss of the neuroprotective ovarian hormone E2 is detrimental to the brain and highlights the importance of studying the effects of long-term estrogen deprivation (surgical menopause and reproductive senescence) on the brain. In fact, we have preliminary data suggesting that E2's post-ischemic regulation of Dkk1 and the Wnt target gene Survivin is lost in long-term E2-deprived rats and that even *basal* levels of these two critical proteins are altered in the hippocampus of non-ischemic sham animals (Scott et al., 2012, *Submitted*). As such, these changes could explain why postmenopausal women, particularly those who enter menopause prematurely due to bilateral oophorectomy, are more susceptible to neurodegenerative disease.

Along these lines, another important, yet controversial, area of study is postmenopausal hormone therapy for the alleviation of menopausal symptoms, cardioprotection, and neuroprotection. Nearly a decade after the Women's Health Initiative (WHI) determined that conjugated equine estrogens ± medroxyprogesterone acetate increased the risk of ischemic stroke (Wassertheil-Smoller et al., 2003) and dementia in women aged 65 and older (Shumaker et al., 2003), despite a wide breadth of basic research suggesting a neuroprotective effect of estrogen, researchers are still hard at work in an attempt to determine the most effective regimen, dose, and route of administration of hormone therapy. Intriguingly, the timing of hormone therapy initiation also seems to be critical for its effectiveness, at least in rodents. In fact, our lab and others have demonstrated that E2's neuroprotective ability is lost in rodents if treatment is delayed 10 weeks following bilateral

ovariectomy or natural aging (Suzuki et al., 2007; Zhang et al., 2009; Zhang et al., 2011). This is consistent with the "critical period hypothesis" of estrogen replacement and the "healthy cell bias" of E2 action, which state that hormone therapy must be initiated at the time of menopause to provide neurological benefit (Brinton, 2008; Harman et al., 2004; Maki, 2006a; Maki, 2006b) [See (Daniel and Bohacek, 2010; Scott et al., 2012) for review of animal studies and clinical studies addressing the critical period hypothesis]. In fact, our lab recently provided a potential mechanism to explain the critical period hypothesis, as unliganded ERα, the principal mediator of E2 neuroprotection, was specifically degraded in the hippocampus of rats that were deprived of ovarian E2 long-term (Zhang et al., 2011). Furthermore, our preliminary data suggest that *delayed* treatment with E2 following surgical menopause is ineffective in preventing the post-ischemic elevation of neurodegenerative Dkk1 or enhancing pro-survival Wnt/β-Catenin signaling (Scott et al., 2012, *Submitted*). Thus, since the brain appears to lose its sensitivity to the beneficial effects of E2 over time following menopause, this underscores the importance of immediate hormone therapy for optimal neurological benefit. As such, more well-designed randomized, controlled clinical trials of hormone therapy are necessary to effectively study this concept in postmenopausal women.

Finally, since elevation of Dkk1 plays a prominent role in neurodegenerative diseases, such as ischemic stroke, Alzheimer's disease, Parkinson's disease, and temporal lobe epilepsy, future studies are needed to explore the potential efficacy of Dkk1 inhibitors as effective therapeutics [See (Caraci et al., 2008) for review]. To date, there are no FDA-approved Dkk1 inhibitors available, and a single commercial Dkk1 inhibitor, WAY-262611, exists for laboratory study. Unfortunately, thus far, only one paper has been published using this inhibitor, which detailed the compound's discovery and demonstrated that oral administration of WAY-262611 promoted trabecular bone growth in ovariectomized rats via inhibition of Dkk1 (Pelletier et al., 2009). Therefore, this compound should be tested in animal models of neurodegenerative disease in order to determine whether it would be a feasible treatment for humans. In addition, neutralizing Dkk1 antibodies have recently become available. In fact, similar to WAY-262611, fully human Dkk1 antibodies have been shown to regulate bone mass *in vivo* (Glantschnig et al., 2010). Furthermore, they have since been utilized to study the role of Dkk1 in glucocorticoid-mediated inhibition of human neural/stem cell progenitor proliferation and differentiation (Moors et al., 2012) and the role of Dkk1 in beta-amyloid-mediated synapse loss in Alzheimer's disease (Purro et al., 2012). Hence, Dkk1 neutralizing antibodies could prove to be an asset to the field, and may also serve as a potential therapeutic treatment for neurodegenerative disease in humans.

In summary, Dkk1 is an antagonist of canonical Wnt signaling, and while it is absolutely critical for embryonic development of the limbs and forebrain, elevation of Dkk1 in the adult brain is associated with neurodegenerative disease. E2 is a neuroprotective ovarian steroid hormone capable of suppressing Dkk1 and activating pro-survival Wnt/β-Catenin signaling *in vivo* following global cerebral ischemia (Zhang et al., 2008), effects implicated to be critical for its neuroprotective actions. Furthermore, E2 is also able to enhance Wnt-independent β-Catenin-mediated transcription *in vitro* through rapid activation of the PI3K-Akt pathway and inactivation of GSK3β (Varea et al., 2009; Varea et al., 2010; Wandosell et al., 2012). While E2 modulation of Dkk1 and Wnt/β-catenin signaling has been well described, comparatively little is known regarding the effects of long-term E2 deprivation (surgical menopause and reproductive senescence) on this important signaling pathway in the brain. Studies on this issue are critically needed, as they may help explain why postmenopausal women have an increased risk of neurodegenerative disease and determine whether this increased risk can be reduced with timely administration of postmenopausal hormone therapy. It is also imperative that preclinical evaluation of Dkk1 inhibitors be fully

explored, especially with regard to their neuroprotective efficacy in neurodegenerative disorders, as they may provide a broad-spectrum therapeutic agent for use in humans.

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Abbreviations

APC	*adenomatous polyposis coli*
CKI	Casein Kinase I
Dkk1	Dickkopf-1
Dvl	Disheveled
E2 or Estrogen	17β-Estradiol
GCI	Global Cerebral Ischemia
Fz	Frizzled
GSK3β	Glycogen Synthase Kinase 3β
HT	Hormone Therapy
JNK	c-Jun N-terminal Kinase
LRP5/6	Low Density Lipoprotein-Related Protein 5/6
LTED	Long-Term Estrogen Deprivation or Long-Term Estrogen-Deprived
MCAO	Middle Cerebral Artery Occlusion
NMDA	N-Methyl-D-Aspartate
PI3K	Phosphatidylinositol 3 Kinase
sFRP	secreted Frizzled-Related Proteins
TCF/LEF	T Cell Factor/Lymphoid Enhancing Factor
Wnt	Wingless
WHI	Women's Health Initiative
WIF-1	Wnt Inhibitory Factor-1
WRE	Wnt Response Element

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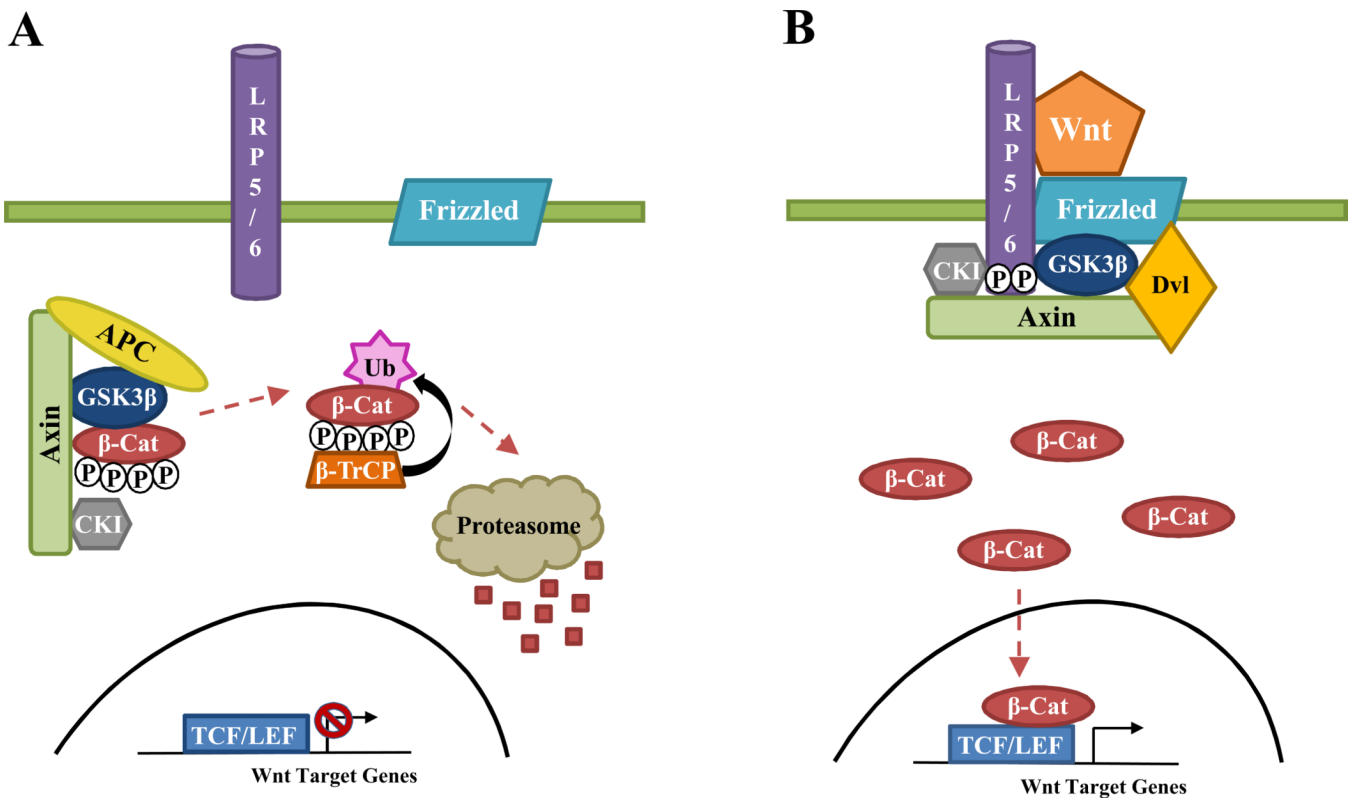

Figure 1. Summary of the Canonical Wnt Signaling Pathway

This figure briefly summarizes canonical Wnt signaling. ***A,*** In the absence of Wnt ligand, cytosolic β-Catenin is phosphorylated, ubiquitinated, and degraded by the proteasome to prevent the expression of Wnt target genes. ***B,*** However, in the presence of Wnt ligand, cytosolic β-Catenin is stabilized and translocates to the nucleus, where it acts as a co-activator and facilitates the transcription of Wnt target genes. Dkk1 antagonizes canonical Wnt signaling by binding to the Wnt co-receptor LRP5/6 and preventing formation of the Wnt-Frizzled-LRP5/6 complex. See text for further details. APC, *adenomatous polyposis coli*, β-Cat, β-Catenin; β-TrCP, Beta-Transducing repeat-Containing Protein; Dvl, Disheveled; CKI, Casein Kinase I; Dkk1, Dickkopf-1; GSK3β, Glycogen Synthase Kinase 3β; LRP5/6, Low Density Lipoprotein-Related Protein 5/6; TCF/LEF, T Cell Factor/ Lymphoid Enhancing Factor; Ub, Ubiquitin; Wnt, Wingless.

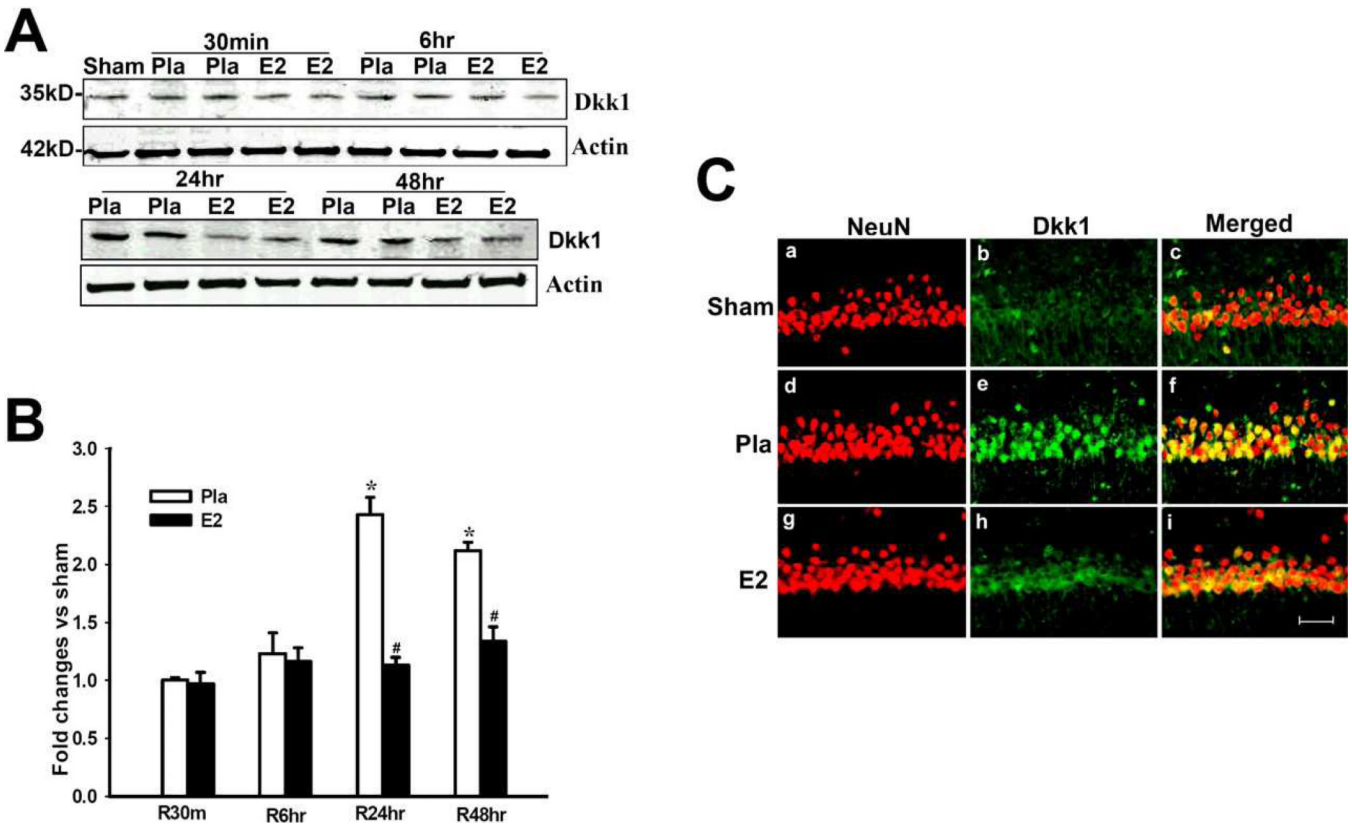

Figure 2. *A,B,* Effect of 17β-estradiol on Dkk1 protein levels in hippocampus CA1 after global cerebral ischemia

Values are mean ± SEM of determinations from five to six individual rats and expressed as fold change versus sham control. Pla, Placebo; R, reperfusion. *p < 0.05 versus sham control; #p < 0.05 versus placebo treatment group. ***Ca–Ci,*** Confocal analysis of NeuN and Dkk1 immunostaining in hippocampus CA1 at 24 h after global cerebral ischemia (magnification, 40X). Scale bar, 50μm. Reprinted, with permission, from *Journal of Neuroscience* (Zhang et al., 2008).

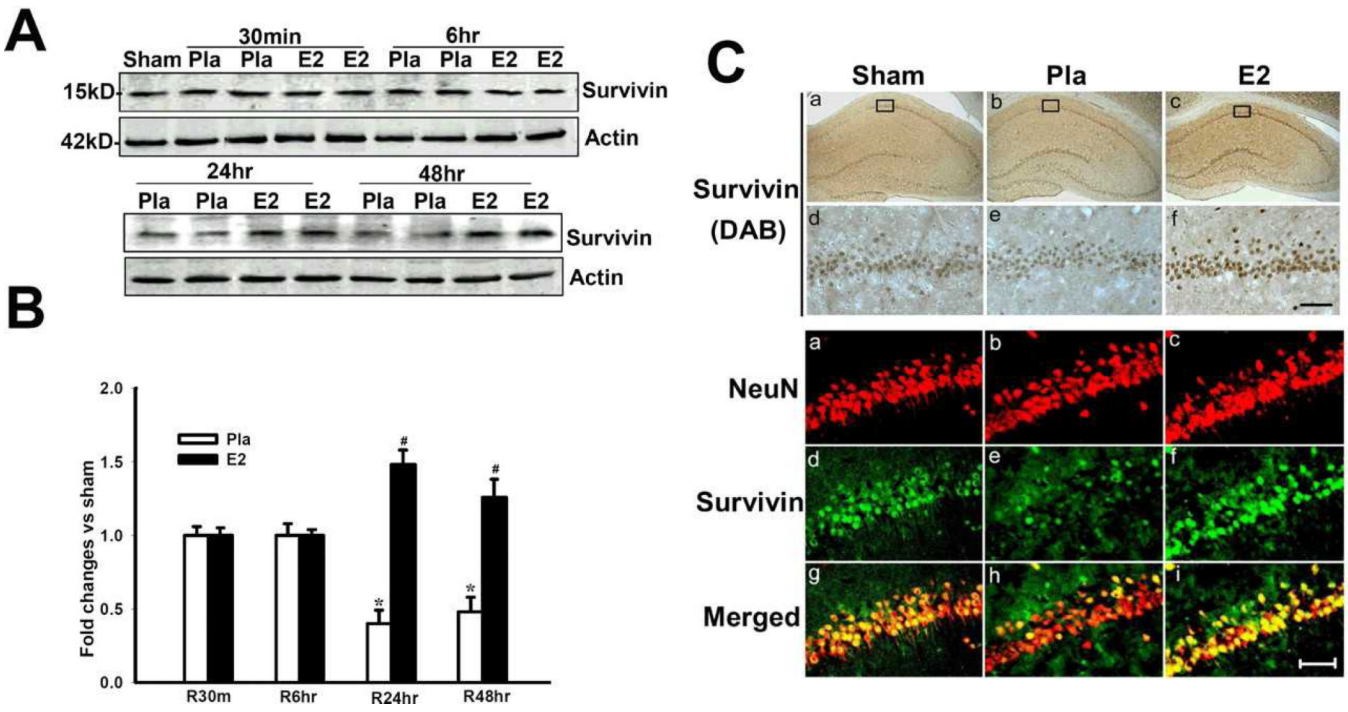

Figure 3. *A,B,* 17β-Estradiol enhances expression of the anti-apoptotic protein Survivin in hippocampus CA1 after global cerebral ischemia

Values are mean ± SEM of determinations from five to six individual rats expressed as fold change versus sham control. Pla, Placebo; R, reperfusion. *p < 0.05 versus sham control; #p < 0.05 versus the Pla group at the same time point. ***C,*** DAB and confocal analysis shows that survivin is induced in NeuN-positive neurons by E2 in hippocampus CA1 at 24 h after global cerebral ischemia. Results are representative of staining observed in five individual animals per group (magnifications, 40X). Scale bars, 50 μm. Reprinted, with permission, from *Journal of Neuroscience* (Zhang et al., 2008).

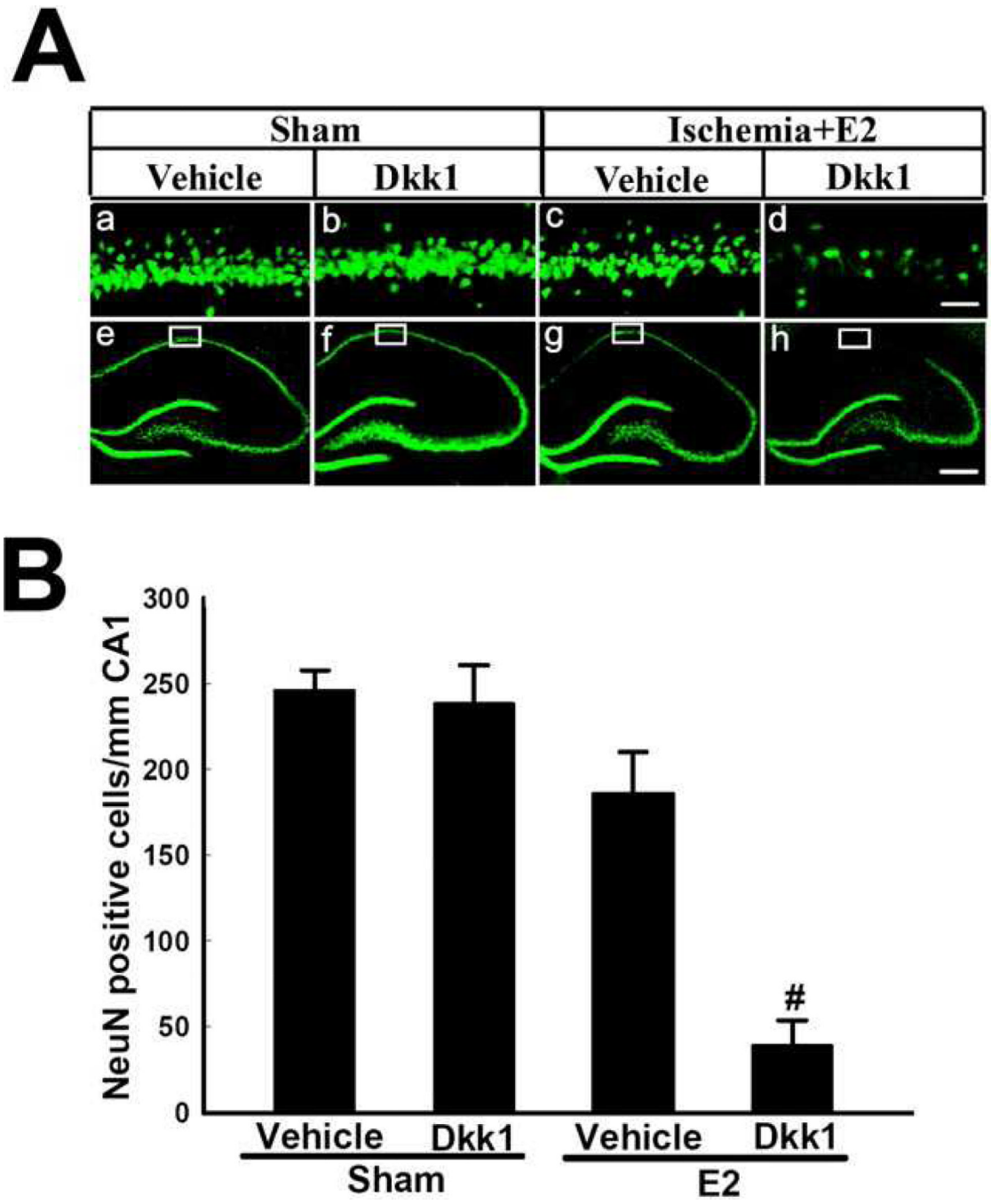

Figure 4. Exogenous Dkk1 Administration Reverses 17β-Estradiol-Induced Neuroprotection after Global Cerebral Ischemia

Dkk1 (5μg/5μl) was administered via intracerebroventricular injection into both lateral ventricles at 12 h after global cerebral ischemia. For a control, separate animals received vehicle into both lateral cerebral ventricles. Additionally, a non-ischemic sham control also received Dkk1 into the lateral ventricles. ***Aa–Ad,*** High-power magnification of NeuN staining in hippocampus CA1 at 7 d in sham animals and after 7 d reperfusion after global cerebral ischemia in E2-treated rats that received vehicle or exogenous Dkk1 in both lateral ventricles. Magnification is 40X. Scale bar, 50μm. ***Ae–Ah,*** Low-power magnifications of representative whole hippocampus sections showing NeuN staining in hippocampus CA1 at

7 d in sham animals and after 7 d reperfusion after global cerebral ischemia in E2-treated rats that received vehicle or exogenous Dkk1 in both lateral ventricles. Magnification is 5X. Scale bar, 200μm. ***B,*** CA1 cell counts of NeuN-positive neurons in all animals show that exogenous Dkk1 had no significant effect on CA1 neuronal cell survival in non-ischemic sham controls, although it significantly reversed E2 neuroprotection in ischemic animals. Values are mean ± SEM of determinations from five to six individual rats. #$p < 0.01$ versus vehicle. Figure adapted from (Zhang et al., 2008).

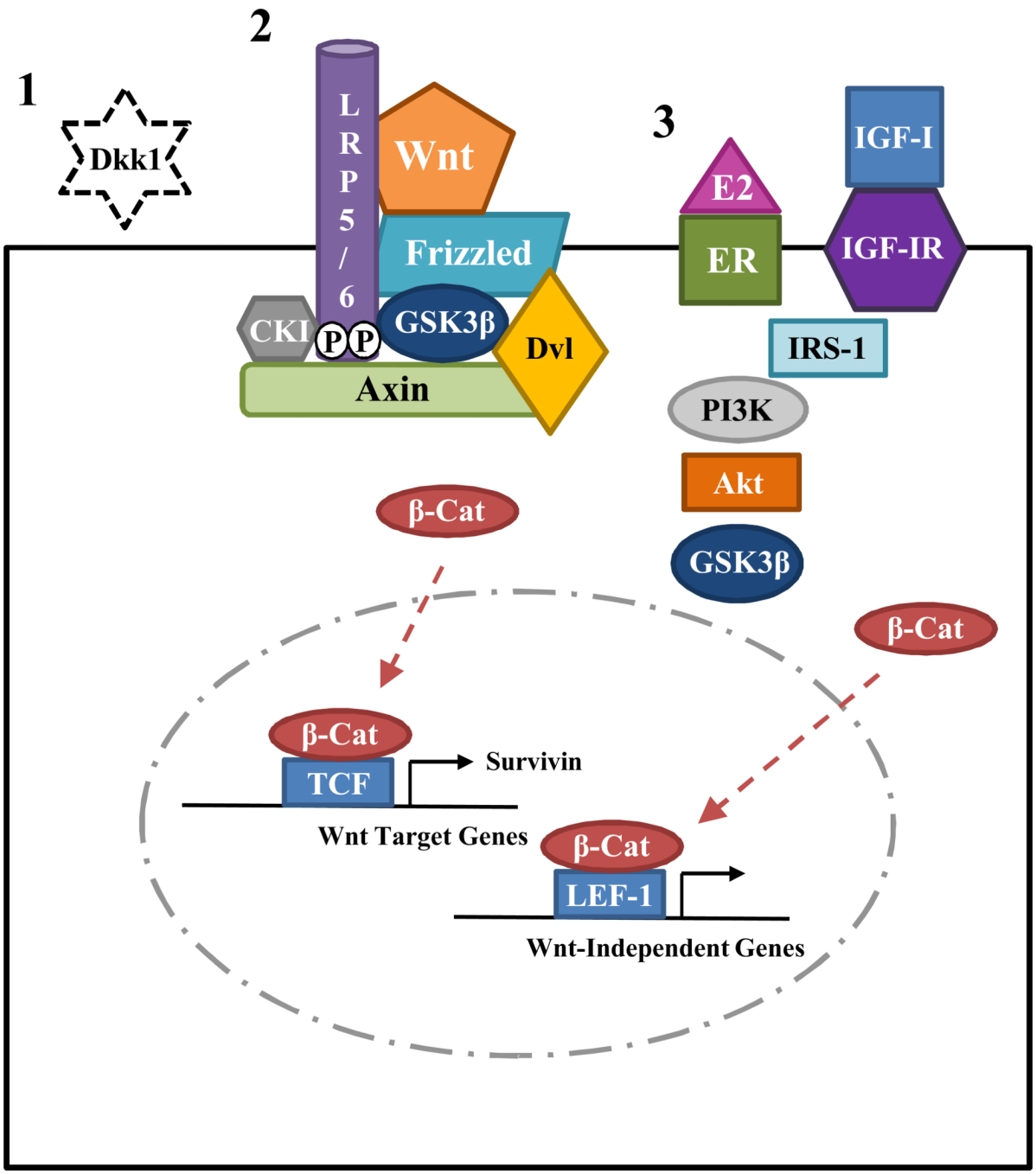

Figure 5. Summary of Estrogen Regulation of Dkk1 and Wnt/β-Catenin Signaling

This figure depicts three currently proposed mechanisms for 17β-estradiol's regulation of Dkk1 and Wnt/β-Catenin signaling in the brain. 1) E2 can suppress expression of the neurodegenerative Wnt antagonist Dkk1 in the hippocampus, particularly following an ischemic insult. 2) E2 can enhance canonical Wnt signaling in the hippocampus, particularly following an ischemic insult, through induction of Wnt3, which leads to expression of pro-survival Wnt target genes, such as Survivin, through β-Catenin and TCF. 3) E2, either acting alone or in concert with IGF-1, can initiate a membrane receptor-mediated PI3K/Akt/GSK3β signaling cascade, which leads to the stabilization of cytosolic β-Catenin and Wnt-independent gene expression through β-Catenin and LEF-1. Akt, A Serine/Threonine Kinase

(also known as Protein Kinase B); β-Cat, Beta-Catenin; CKI, Casein Kinase 1; Dkk1, Dickkopf-1; Dvl, Disheveled; E2, 17β-Estradiol; ER, Estrogen Receptor; GSK3β, Glycogen Synthase Kinase 3 Beta; IGF-I, Insulin-like Growth Factor-1; IGF-IR, Insulin-like Growth Factor-1 Receptor; IRS-1, Insulin Receptor Substrate 1; LEF-1, Lymphoid Enhancing Factor 1; LRP 5/6, Low-Density Lipoprotein 5/6; PI3K, Phosphatidylinositol 3 Kinase; TCF, T Cell Factor; Wnt, Wingless.

Table 1
Summary of Evidence Associating Dkk1 with Neurodegenerative Disease

This table briefly summarizes the major research findings to date linking the Wnt antagonist Dkk1 to four neurodegenerative diseases: stroke, temporal lobe epilepsy, Alzheimer's disease, and Parkinson's disease. See text for further details. Aβ, β-Amyloid; Dkk1, Dickkopf-1; GCI, Global Cerebral Ischemia; ET-1, Endothelin-1; MCAO, Middle Cerebral Artery Occlusion; MPP^+, 1-methyl-4-phenylpyridinium;NMDA, N-Methyl-D-Aspartate; 6-OHDA, 6-Hydroxydopamine.

References	Finding	Model System	Neurodegenerative Disease
(Cappuccio et al., 2005)	Dkk1 ↑ after NMDA administration and potentiates NMDA excitotoxicity	Cultured Cortical Neurons	Stroke
(Scali et al., 2006)	Dkk1 causes cell death and tau hyperphosphorylation	Cultured Cortical Neurons	Stroke
(Cappuccio et al., 2005; Zhang et al., 2008)	Dkk1 ↑ in hippocampus after GCI	Gerbils and Rats	Stroke
(Mastroiacovo et al., 2009)	Dkk1 ↑ in cortex after ET-1 infusion and permanent MCAO	Rats	Stroke
(Scali et al., 2006)	Stereotaxic injection of Dkk1 into the brain causes cell death	Rats	Stroke
(Cappuccio et al., 2005)	↓ of Dkk1 with anti-sense oligonucleotides attenuates cell death after GCI	Gerbils	Stroke
(Mastroiacovo et al., 2009)	Doubleridge mice with ↓ Dkk1 have smaller cortical infarcts after MCAO	Mice	Stroke
(Seifert-Held et al., 2011)	Serum Dkk1 ↑ after ischemic stroke	Humans	Stroke
(Busceti et al., 2007)	Dkk1 ↑ after kainate-induced seizures	Rats	Epilepsy
(Busceti et al., 2007)	↓ of Dkk1 with anti-sense oligonucleotides protects against kainate damage	Rats	Epilepsy
(Busceti et al., 2007)	Dkk1 ↑ in temporal lobe epilepsy brain specimens	Humans	Epilepsy
(Caricasole et al., 2004; Purro et al., 2012)	Aβ ↑ Dkk1	Cultured Cortical Neurons	Alzheimer's Disease
(Caricasole et al., 2004)	↓ of Dkk1 with anti-sense oligonucleotides protects against Aβ toxicity and prevents tau hyperphosphorylation	Cultured Cortical Neurons	Alzheimer's Disease
(Caricasole et al., 2004)	Dkk1 ↑in post-mortem Alzheimer's disease brain specimens and co-localizes with neurofibrillary tangles	Humans	Alzheimer's Disease
(Rosi et al., 2010)	Dkk1 ↑ in the brain of transgenic models of Alzheimer's disease, particularly surrounding amyloid deposits and in neurons with hyperphosphorylated tau	Mice	Alzheimer's Disease
(Purro et al., 2012)	Dkk1 rapidly and reversibly ↓ synapse size and number	Rat Hippocampal Neurons	Alzheimer's Disease
(Purro et al., 2012)	Dkk1 neutralizing antibodies suppress synapse loss related to acute Aβ exposure	Mouse Hippocampal Slices	Alzheimer's Disease
(L'Episcopo et al., 2011)	Dkk1 reversed astrocyte-induced neuroprotection from MPP^+ toxicity	Mouse Astrocyte-Neuron Co-Cultures	Parkinson's Disease
(Dun et al., 2012)	Dkk1↑ after 6-OHDA administration and potentiates 6-OHDA neurotoxicity in substantia nigra	Rats	Parkinson's Disease