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The PSD-95/nNOS Complex: New Drugs for Depression?

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Abstract

Drug treatment of major depressive disorder is currently limited to the use of agents which influence monoaminergic neuronal transmission including inhibitors of presynaptic transporters and monoamine oxidase. Typically improvement in depressive symptoms only emerges after several weeks of treatment, suggesting that downstream neuronal adaptations rather than the elevation in synaptic monoamine levels are responsible for antidepressant effects. In recent years, the NMDA receptor has emerged as a promising target for treating CNS disorders including stroke, pain and depression. In this review, we outline the molecular mechanisms underlying NMDA receptor signaling in neurons and in particular provide an overview of the role of the NMDAR/PSD-95/nNOS complex in CNS disorders. We discuss novel drug developments made that suggest the NMDAR/PSD-95/nNOS complex as a potential target for the treatment of depression. The review also provides examples of how PDZ-based protein-protein interactions can be exploited as novel drug targets for disease.
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1. Introduction

Mood disorders, including bipolar and major depressive disorders, are common neuropsychiatric illnesses in industrialised countries. Depression is estimated to become the leading cause of disability worldwide by the year 2030, with approximately 100 million suggested to suffer from anxiety and depression within Europe alone (WHO, 2005). Typical clinical features of depression include feelings of worthlessness, low self-esteem, irritability, recurrent thoughts of death or suicide and a marked loss of interest, in addition to multiple cognitive and sleep/wake abnormalities (American Psychiatric Association, 2000).

Since the 1960's, several antidepressants have been developed that target the monoaminergic systems, including dopamine, serotonin and noradrenaline. These neuronal pathways project widely to the limbic, striatal and prefrontal cortical areas, and have been broadly implicated in the behavioural and visceral manifestations of mood disorders. The discovery of iproniazid (a monoamine oxidase inhibitor, MAOI) and imipramine (a tricyclic antidepressant) for the treatment of depression led to the monoamine hypothesis of depression, linking a possible deficiency of noradrenaline and/or serotonin to this disorder (Schildkraut, 1965). These drugs improve the symptoms of patients however they target several neurotransmission systems and produce adverse effects. In addition, selective serotonin reuptake inhibitors (SSRIs) have been developed, which block the presynaptic transporter SERT and promote the reuptake of serotonin, thereby increasing neurotransmitter availability at the synaptic cleft (Wong and Licinio, 2004) (for a comprehensive summary of antidepressants, see Table 1).

After more than 50 years of research, the molecular mechanisms underlying depression have not been clearly elucidated. A 6-year prospective study enrolling more than 3,500 patients in the US (STAR*D) showed that only one third of patients receiving antidepressants achieved remission with initial treatment. Those patients where antidepressant therapies did not show efficacy after initial treatment were more prone to successive treatment failures (Nelson, 2006). Furthermore, improvement in symptoms emerges only after several weeks of treatment, suggesting that downstream neuronal adaptations rather than the elevation in synaptic monoamine levels itself are responsible for therapeutic effects. Thus, although monoaminergic systems have an important role in the pathophysiology and treatment of mood disorders, other neurotransmitters, such as glutamate, that regulate synaptic and neural plasticity may also play a central role in the neurobiology and management of depression. In support for a role of aberrant excitatory neurotransmission in depression, a recent genome-wide association study has revealed SLC6A15, which codes for a neuronal Na⁺-dependent amino acid transporter (Broer, 2006), as a susceptibility gene for depression (Kohli et al., 2011). The data indicate that lower SLC6A15 expression in the hippocampus could increase stress susceptibility by altering neuronal integrity and excitatory neurotransmission in this brain region (Kohli et al., 2011). In addition, a role for glutamate neurotransmission in the pathophysiology of major depressive disorder and as a potential drug target for new therapeutics has been previously proposed (Sanacora et al., 2008, Hashimoto, 2009, Skolnick et al., 2009). A number of studies have also shown that regulation of the glutamatergic system, in particular, the NMDA (N-methyl-D-aspartate) receptor subtype, may provide means for development of effective and fast-acting antidepressant therapies (Berman et al., 2000, Zarate et al., 2006b). Here, we provide an overview of a downstream signaling pathway linked to this receptor that may be specifically targeted for the development of novel antidepressant therapies.
2. The NMDAR/PSD-95/nNOS complex

The family of glutamate receptors is divided into ionotropic subtypes comprising α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and NMDA receptors and metabotropic subtypes mGluR1-8 based on sequence homology, pharmacology, and electrophysiological properties (Scannevin and Huganir, 2000). The NMDA receptor is widely distributed in mammalian brain. Activation of the NMDA receptor leads to Ca\(^{2+}\) influx as well as regulation of other signaling pathways including neuronal nitric oxide synthase (nNOS, also called NOS-1). The activation of nNOS via NMDA receptors requires interaction with the scaffold protein PSD-95 (postsynaptic density 95 kDa), which forms an NMDAR/PSD-95/nNOS complex (Figure 1).

2.1. The NMDA receptor

NMDA receptors are tetrameric and typically contain two NR1 and two NR2 subunits (also called GluN1 and GluN2). Opening of the NMDA receptor channel requires the binding of both glutamate on the NR2 subunit and the co-agonist glycine on NR1 (Dingledine et al., 1999, Erreger et al., 2004). Additional binding sites include a site within the channel at which use-dependent antagonists such as ketamine and MK-801 can bind. NMDA receptors can also contain NR3 subunits that modulate receptor properties by, for example, reducing both whole-cell currents and single-channel conductance (Dingledine et al., 1999). At a neuronal resting membrane potential of -65mV, NMDA receptors undergo channel block by extracellular Mg\(^{2+}\). The AMPA receptor-induced depolarisation of neurons allows lifting of the voltage-dependent block by Mg\(^{2+}\), via influx of mainly Ca\(^{2+}\) and to a lesser extent Na\(^{+}\). The Ca\(^{2+}\) influx triggers a variety of intracellular signaling cascades including activation of a Ca\(^{2+}\)/calmodulin complex, which in turn stimulates nNOS leading to the production of nitric oxide (Figure 1). Over-activation of the NMDA receptor is thought to cause excitotoxicity due to excessive Ca\(^{2+}\) influx, however studies show that Ca\(^{2+}\) blockers alone are not protective against this process (Franke et al., 1996, Horn and Limburg, 2000, Horn et al., 2001, Aarts and Tymianski, 2003). These findings have led to suggestions that the activation of additional intracellular cascades coupled to the NMDA receptor, such as nitric oxide, are also important in excitotoxicity (Aarts and Tymianski, 2003).

2.2. The postsynaptic density protein PSD-95

The postsynaptic density (PSD) is a membrane-associated mega-organelle specialised for postsynaptic signal transduction and processing (Feng and Zhang, 2009). The PSD is located at the head of dendritic spines and is a disk-like structure of approximately 200-800 nm wide and 30-50 nm thick (Feng and Zhang, 2009) occupying approximately 10% of the surface area of the spine (Hering and Sheng, 2001). Importantly, at the PSD, synaptic-expressed proteins are aligned with the presynaptic active zone (Hering and Sheng, 2001). There are four PSD family members classified according to their molecular weight, including, PSD-95 (SAP-90), PSD-93 (Chapsyn-110), synaptic associated proteins 97 kDa and 102 kDa (SAP-97 and SAP-102, respectively) (Figure 2). Briefly, SAP-97 is found in the pre- and postsynaptic compartments, whereas PSD-95, PSD-93 and SAP-102 are found at the postsynaptic membrane of excitatory synapses (Kalia and Salter, 2003). The postsynaptic-expressed PSD proteins are located close to the membrane at a mean distance of 12 nm (Kim and Sheng, 2004), where PSD-95 and PSD-93 form multimers mediated by N-terminal "head to head" interactions (Hsueh et al., 1997, Hsueh and Sheng, 1999, Christopherson et al., 2003). The family of PSD proteins is made of three PDZ (PSD-95/DlgA/Zo-1) domains, a SH3 (Src homology 3) domain and a
GK (guanylate kinase) domain, which has lost its catalytic activity (Figure 2). Of particular interest, PSD-95 interacts with both ionotropic and metabotropic glutamate receptors via protein-protein interactions and plays a role in their precise assembly and spatial organisation as well as coupling these receptors to downstream signaling events (Scannevin and Huganir, 2000).

2.3. Neuronal nitric oxide synthase (nNOS)
Nitric oxide synthases are divided into three major isoforms: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) (Figure 3A). The human neuronal isoform (nNOS) is a 1434 amino acid protein of 160.8 kDa. The gene encoding nNOS is located on chromosome 12 (12q24.2-12q24.3), incorporates 29 exons and shows sequence conservation through many species (Alderton et al., 2001) (Figure 3B). nNOS has been identified in developing and mature neurons, but is also present in skin and bronchial epithelium (Hobbs et al., 1999, Guix et al., 2005). In skeletal muscles (Alderton et al., 2001), nNOS binds to α1-syntrophin and caveolin-3 to form a complex with sarcolemmal dystrophin (Venema et al., 1997, Christopherson et al., 1999).

2.4. Expression of the NMDAR/PSD-95/nNOS complex in the CNS
The NR1 subunit is ubiquitously expressed in the adult human brain, while the NR2A-D subunits display a region specific distribution (Karolewicz et al., 2005). For example, NR2C is strongly expressed in the locus coeruleus, while NR2A and NR2B are found at very low levels in this brain region (Karolewicz et al., 2005). In contrast, modest levels of NR2A and high levels of NR2B and NR2C are found in all subfields of the hippocampus, including pyramidal neurons, granule cells and polymorphic hilar cells (Law et al., 2003, Karolewicz et al., 2005). NR1 and NR2B also undergo developmental regulation, respectively increasing and decreasing after the neonatal period whereas NR2A levels remain unchanged (Law et al., 2003). Importantly, PSD-95 is widely expressed in the human brain and overlaps with NMDAR distribution, including regions implicated in mood regulation such as the frontal cortex (Toro and Deakin, 2005, Beneyto and Meador-Woodruff, 2008, Feyissa et al., 2009), amygdala (Karolewicz et al., 2009), hippocampus (Toro and Deakin, 2005) and striatum (Kristiansen and Meador-Woodruff, 2005). High protein levels of nNOS are also found in several regions where both NMDAR and PSD-95 are expressed (Blum-Degen et al., 1999). In particular, nNOS is found in hippocampal pyramidal cells and interneurons of the CA1 and CA3 layers and in granule cells of the dentate gyrus in both human and rodent brains (Blackshaw et al., 2003; Law et al., 2003). Interestingly, hippocampal levels of nNOS are greater in mouse than in rat, with a prominent expression in pyramidal layers of CA1 and CA3 (Burette et al., 2002, Blackshaw et al., 2003). The association of nNOS with NMDARs, via PSD-95 (Christopherson et al., 1999), plays an important role in a range of normal neuronal functions including synaptic plasticity and learning and memory (Garthwaite et al., 1988, Jaffrey and Snyder, 1995), as well as pathophysiological disorders of the brain such as stroke and pain (Aarts et al., 2002, Sun et al., 2008, Florio et al., 2009, Zhou et al., 2010). With regard to depression, post-mortem studies revealed region specific changes in NR1, NR2A-C, PSD-95 and nNOS expression levels in depressed patients when compared to matched controls, which support the hypothesis that the NMDAR/PSD-95/nNOS complex is altered in major depressive disorder (Table 2).
3. PDZ domain interactions in the NMDAR/PSD-95/nNOS complex

The family of PDZ domain containing proteins comprise more than 150 members that are, in most part, otherwise unrelated (www.uniprot.org). PDZ domains are 80-90 amino acids long and are the most abundant protein domain in eukaryotes (Feng and Zhang, 2009). These domains contain a canonical peptide binding groove typically composed of six β-strands (βA to βF) and two α-helices (αA and αB), which recognise a short stretch of residues at the carboxyl terminal (ct) of target proteins, known as PDZ motifs (Feng and Zhang, 2009). Thus, PDZ domains can be considered as intracellular domains or ‘receptors’ that can be filled by a PDZ motif or ‘ligand’ (Dev, 2004). PDZ-based interactions play a diverse role in cellular function, including the trafficking and membrane expression of synaptic receptors, the targeting of kinases and E3 ligases for receptor phosphorylation and ubiquitination respectively, and maintenance of the synaptic cyto-architecture and morphology (Kim and Sheng, 2004, Feng and Zhang, 2009).

PSD-95 contains a SH3 domain, a GK domain and three PDZ domains. The PDZ domains 1 and 2 of PSD-95 are separated by only a few residues, creating a rigid bond that may restrain interdomain flexibility. These two PDZ domains can have distinct or overlapping target-binding proteins (Feng and Zhang, 2009). In contrast, the PDZ3 of PSD-95 is located at the carboxyl terminal end of the protein and has a set of interacting proteins that are distinct from PDZ domains 1 and 2 (Lim et al., 2002). The N-palmitoylation of PSD-95 at Cys-3 and Cys-5 residues (El-Husseini et al., 2000) is essential for enhanced synaptic responses (Schnell et al., 2002) and the trafficking of PSD-95 to the plasma membrane (El-Husseini et al., 2000). With its several domains, PSD-95 is often referred to as a scaffold protein, which localises and traffics various receptors, cell adhesion molecules, ion channels, kinases and phosphatases to the synaptic membrane. PSD-95 also interacts with a variety of adaptor and cytoskeleton proteins, molecular motors, as well as protein synthesis machinery (Scannevin and Huganir, 2000, Feng and Zhang, 2009). Thus, PSD-95 plays a central role in receptor surface delivery, endocytosis and subcellular localisation, by which it can regulate the subunit composition and channel conductance of receptors expressed at the synapse, in particular NMDA receptors (Kim and Sheng, 2004, Kalia et al., 2006). To date, more than 40 proteins have been identified to interact with PSD-95, a majority of which are localised at the PSD (Yamauchi, 2002) (Figure 4A).

Importantly, NMDA receptor subunits contain PDZ motifs that can interact with several proteins located in the cytosol (Figure 4B). In particular, the ct of NR2A, NR2B or NR1 subunits contains a PDZ motif (-ESDV, -ESDV and -STVV respectively) that bind PDZ1 or PDZ2 of PSD-95, but not PDZ3 (Kornau et al., 1995, Niethammer et al., 1996). In addition, PSD-95 (PDZ2) also interacts with nNOS (Brenman et al., 1996). Specifically, nNOS contains two non-overlapping binding sites: (i) a canonical PDZ domain (residues 1-99) that binds PDZ motifs (Chanrion et al., 2007, Manivet et al., 2000, Jaffrey et al., 1998, Firestein and Bredt, 1999, Riefler and Firestein, 2001, Lin et al., 2003, Saitoh et al., 2004) and (ii) an “internal” PDZ motif (residues 100-130) that binds PDZ domains of other proteins (Hillier et al., 1999, Christopherson et al., 1999) (Figure 4B). This internal PDZ motif (residues 100-130) of nNOS is arranged in a flexible, transient β-sheet structure and is usually referred to as β-hairpin or β-finger. The PSD-95 (PDZ2) interacts with the nNOS residues 108-111 (-ETTF-, an internal PDZ motif), which transforms the flexible β-hairpin into a rigid and stable β-sheet (Tocchio et al., 2000). Then NOS splice variants that lack a PDZ motif do not associate with PSD-95, suggesting a primary role for the internal PDZ motif in mediating subcellular localisation of nNOS.
(Brenman et al., 1996). Taken together, these PDZ-based interactions create a ternary protein complex composed of NR2B(-ESDV)/PSD-95(PDZ1) and PSD-95(PDZ2)/nNOS(-ETTF-) interactions (Christopherson et al., 1999) (Figure 1).

4. Current drugs used to target NMDAR and NOS in depression

Delayed action of existing antidepressant agents suggest they act on substrates upstream of the targets that are ultimately responsible for their effects. The delay associated with the onset of action of SSRIs is thought to arise from slow adaptation to presynaptic 5HT1B/1D and somatodendritic 5HT1A autoreceptors, which undergo desensitization upon chronic administration of antidepressants (Cryan and Leonard, 2000). Recent studies also suggest further neuronal adaptation including increases in brain derived neurotrophic factor (BDNF) and neurogenesis in the dentate gyrus of the hippocampus associated with chronic antidepressant administration and the delayed onset of antidepressant action (Duman, 2004, Krishnan and Nestler, 2008). In addition, other neurotransmitters have been proposed in mediating the delayed effects of monoaminergic-based antidepressants. In this context, the glutamatergic system, and in particular adaptive changes to the NMDA receptor complex have been proposed to serve as a common pathway for neurobiological events which underlie antidepressant action. Antidepressant treatments may induce changes to the ligand binding properties of the NMDAR complex (Petrie et al., 2000, Paul and Skolnick, 2003, Pittenger et al., 2007) in addition to effecting a reduction in depolarization-evoked presynaptic glutamate release (Bonanno et al., 2005). Such effects appear to occur in a regional and time dependent fashion in keeping with the clinical actions of antidepressants.

Further support for a role of the NMDAR as a locus of antidepressant activity stems from evidence that NMDAR antagonists produce antidepressant effects both clinically and in several animal models of antidepressant action (Skolnick et al., 2009). Two clinical studies have reported a rapid improvement in depressive symptoms after intravenous administration of ketamine, a non-competitive NMDA receptor antagonist at a dose of 0.5mg/kg (Berman et al., 2000, Zarate et al., 2006b). Additional reports have more recently confirmed these effects. For example, using a similar treatment regime to earlier investigations, an open-label study demonstrated that ketamine reduced depressive symptoms in treatment-resistant patients with a significantly higher response in individuals with a confirmed family history of alcohol abuse when compared to patients with no family history (Phelps et al., 2009). Moreover, studies have shown a rapid and robust antidepressant action of ketamine in treatment-resistant depressed patients and determined that suicidal ideation was significantly reduced (Price et al., 2009, DiazGranados et al., 2010). In the context of suicide risk, which requires rapid intervention, a small open-label trial in an emergency department used ketamine as a single intravenous bolus of 0.2 mg/kg administered over 1-2 min. Symptoms of depression and suicidal ideation decreased significantly within 40 min, with no evidence of recurrence during the 10-day follow-up, as determined by the Montgomery Asberg depression rating scale (MADRS) (Larkin and Beautrais, 2011). Of interest, a case series investigated the possibility of oral administered ketamine (1.25 mg/kg) as add-on therapy for depression. In this small study, ketamine was well-tolerated and the drug showed rapid improvement in symptoms; a caveat of this study was however the low number of patients recruited (Paslakis et al., 2010). Finally, a retrospective study reported synergistic effects of ketamine when
administered as an anaesthetic agent for electroconvulsive therapy (Kranaster et al., 2011). Whilst beneficial effects were observed for up to 7-10 days (Zarate et al., 2006b, Larkin and Beautrais, 2011), the clinic use of ketamine has been hampered due to its adverse effects including psychosis and psychomotor stimulation (Berman et al., 2000). Ketamine also caused brief hypertensive episodes and tachycardia or bradycardia after repeated doses (aan het Rot et al., 2010).

Memantine, a low-to-moderate-affinity non-competitive NMDA receptor antagonist licensed for the treatment of Alzheimer’s disease, has also been tested as a potential antidepressant. This drug showed no improvement in clinical symptoms when administered at a maximum dose of 20mg daily in patients with major depressive disorder (Zarate et al., 2006c) but revealed a significant reduction in depressive symptomatology when the dose was titrated up to 40mg/day in a small open-label trial (Ferguson and Shingleton, 2007). Two other agents have been investigated for the treatment of bipolar depression with mixed results. Lamotrigine (licensed for the treatment of epilepsy and bipolar disorder) and riluzole (licensed for the treatment of amyotrophic lateral sclerosis) are thought to modulate glutamate release via inhibition of use-dependent Na\(^+\) channels, Ca\(^{2+}\) channels and effects on K\(^+\) channels (Sanacora et al., 2003, Pittenger et al., 2008). Riluzole additionally plays a role in the clearance of glutamate through enhancement of reuptake by astrocytes and in AMPAR trafficking by increasing membrane insertion of GluR1 (GluA1) and GluR2 (GluA2) (Zarate et al., 2006a, Pittenger et al., 2008, Machado-Vieira et al., 2009). A study using riluzole at a daily dose of 50-200mg was suggested to improve symptoms as measured by the MADRS (Zarate et al., 2005). Notably, this study used an open-label and non-randomised design, with a small sample of patients examined. Moreover, lamotrigine at doses ranging from 50-400mg daily did not improve symptoms in the acute treatment of bipolar depression in five double-blind and placebo-controlled clinical trials (Calabrese et al., 2008). Thus despite the antidepressant effects of ketamine, not all clinical trials of NMDAR antagonists or agents which influence the synaptic availability of glutamate have yielded such dramatic results. A recent study of the NR2B selective antagonist traxoprodil (CP 101606) in treatment-resistant depression is particularly noteworthy as patients who had failed to respond to the SSRI paroxetine showed a greater improvement in MADRS scores following a single infusion of traxoprodil with continued paroxetine when compared to infusion placebo controls (Preskorn et al., 2008). The response rate was 3-fold higher in traxoprodil patients when compared to placebo controls and was maintained in over 30% of patients for up to 30 days post-infusion. Although more rigorous clinical testing will be required the results lend support to the hypothesis that reduction of NMDA receptor function can produce rapid antidepressant effects in treatment-resistant patients. Taken together, it appears that the regulation of glutamate levels and of NMDAR function may offer a therapeutic method for treatment of depression, provided that associated psychosis and psychomotor stimulation can be avoided.

To date the mechanisms mediating the rapid and sustained antidepressant actions of NMDAR antagonists have not been resolved. A pharmaco-MRI study measuring the blood oxygenation level-dependent (BOLD) signal on healthy participants demonstrated that ketamine increased BOLD activity in regions involved with the processing of emotions, including the cingulate gyrus and the hippocampus. In contrast, decreases in activity were observed in the ventromedial frontal cortex (Deakin et al., 2008). Some of the effects of ketamine may be attributable to glutamate release since pre-treatment with lamotrigine blocked many of the BOLD signal changes and the symptoms evoked by ketamine (Deakin et al., 2008). With regard to
depression, preclinical studies have reported that AMPA receptor activation is required for the antidepressant-like actions of ketamine (Maeng et al., 2008). A role for nitric oxide and cGMP dependent protein kinase, under the regulation of the NMDAR, in AMPA receptor trafficking and synaptic plasticity has been reported (Rameau et al., 2007, Serulle et al., 2007), linking the various signaling components most likely implicated in the antidepressant activity associated with NMDAR antagonists. Moreover acute administration of ketamine (10 mg/kg) activates the mammalian target of rapamycin (mTOR) signaling pathway in the prefrontal cortex of rats increasing the expression of synaptic proteins and the density of dendritic spines in tandem with a rapid antidepressant response in behavioural tests (Li et al., 2010, Li et al., 2011). mTOR is a serine/threonine kinase that has been implicated in activity-dependent synaptic plasticity and controls the synthesis of proteins that are required for new synapse formation in neuronal dendrites and spines (Hoeffer and Klann, 2010, Li et al., 2011). The mTOR pathway appears to be compromised in the prefrontal cortex of depressed patients, as demonstrated by a decrease of mTOR and its downstream signaling proteins (Jernigan et al., 2011). Recent evidence additionally supports a role for BDNF in the rapid antidepressant effects observed in mice following ketamine administration. Specifically, the blockade of the NMDARs with ketamine (3 mg/kg) causes synthesis of BDNF in the hippocampus and ketamine antidepressant-like effects are absent in BDNF and BDNF receptor (TrkB) knockout animals (Autry et al., 2011).

Since NMDA couples to nNOS and NMDAR antagonists possess antidepressant properties, the downstream inhibition of nNOS has also been investigated as an experimental approach to produce antidepressant effects lacking the problems associated with direct inhibition of the receptor. NOS inhibitors display promising antidepressant activity in preclinical studies (Wegener and Volke, 2010). In a mouse model of antidepressant activity, specifically a forced swim test (FST), the nitric oxide synthase inhibitors N\(^\text{G}\)-nitro-L-arginine (L-NA), N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA), N\(^\text{G}\)-nitro-L-arginine methylester (L-NAME) and 1-[2-(trifluoromethyl)phenyl] imidazole (TRIM) demonstrated the same efficacy as the tricyclic antidepressant imipramine (Harkin et al., 1999, Volke et al., 2003). Furthermore, behavioural effects were dose-dependent and stereoselective and reversed by co-treatment with the NOS substrate L-arginine consistent with involvement of nitric oxide in the antidepressant response. Inhibitors of nNOS elicit antidepressant activity when administered alone in these tests or, in low doses, augment the activity of sub-threshold doses of antidepressant drugs (Harkin et al., 2004). By contrast to NMDA receptor antagonists, NOS inhibitors are devoid of locomotor stimulatory properties (Harkin et al., 1999, Harkin et al., 2004). In other preclinical investigations, nNOS deletion or treatment with the NOS inhibitors 7-nitroindazole (7-NI) or TRIM prevented chronic mild stressor (CMS)-induced depression-related behavioural changes in mice further confirming the antidepressant potential of NOS inhibition (Zhou et al., 2007, Mutlu et al., 2009). Mice exposed to CMS overexpressed hippocampal nNOS and showed impaired neurogenesis in the hippocampus (Zhou et al., 2007). CMS-induced behavioural deficit as measured by increased immobility in the tail suspension test and impairment in hippocampal neurogenesis were reversed in mice lacking the nNOS gene or mice treated with 7-NI. Moreover disrupting hippocampal neurogenesis blocked the antidepressant effects of 7-NI in the model. Such findings suggest that nNOS over expression and reduced neurogenesis in the hippocampus are associated with stress-induced depression and that inhibition of nNOS signaling in the brain represents a novel approach to antidepressant treatment. However, despite these promising studies, efforts in
synthesising highly selective nNOS inhibitors have been difficult and have limited the development of such drugs (Vallance and Leiper, 2002).

5. Disruption with blocking peptides and small-molecule inhibitors

Disruption of the NMDAR/PSD-95/nNOS complex represents an alternative approach to specifically block NMDAR signaling coupled to nNOS, which may allow for a better side effect profile. To date, however PDZ-based interactions represent a drug target that still remain largely untapped (Dev, 2004). A common method used to disrupt PDZ-based interactions consists of using a blocking peptide or compound that competitively binds to the PDZ domain and prevents PDZ motif(s) from interacting (Aarts et al., 2002, Soriano et al., 2008, Sun et al., 2008, Fan et al., 2009, Florio et al., 2009, Zhou et al., 2010). The first report to demonstrate disruption of the NMDAR/PSD-95 interaction by use of a competitive blocking peptide showed a protective effect in the middle cerebral artery occlusion (MCAO) model of stroke without altering synaptic activity or calcium influx (Aarts et al., 2002). In this study, the last nine C-terminal residues of NR2B (KLSSIESDV) were fused to the cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) Tat protein to create Tat-NR2B9c (YGRKKRRQRRRKLSIESDV). Using this peptide, the study confirmed that PSD-95 is required for coupling of the NMDAR to nitric oxide and Ca\(^{2+}\)-signaling, which plays a role in neuronal excitotoxicity (Sattler et al., 1999). Since PSD-95 interacts with many proteins via its PDZ domains (Kim and Sheng, 2004), the binding of Tat-NR2B9c to the PDZ domains of PSD-95 could in theory alter several interactions and thus cause side effects. Surprisingly, however, inhibition of NMDAR/PSD-95 interaction by Tat-NR2B9c was not reported to cause any overt behavioural changes (Aarts et al., 2002). In agreement, a later study examined the long-term neurological outcomes of MCAO rats treated with Tat-NR2B9c and reported no physical, sensorimotor or cognitive adverse effects (Sun et al., 2008).

In recent years, disruption of PSD-95/nNOS rather than NMDAR/PSD-95 has been preferred in order to block downstream NO-mediated excitotoxicity without blocking NMDAR-mediated excitable neurotransmission. Specific disruption of the PSD-95/nNOS interface has been achieved with cell fusion proteins based on the first 300 residues of nNOS (nNOS\(_{1-300}\)) (Cao et al., 2005, Florio et al., 2009, Zhou et al., 2010). This nNOS\(_{1-300}\) protein fragment contains the PDZ domain and β-finger of nNOS and can act as a competitive inhibitor of the interaction between the PDZ domain of nNOS and the PDZ2 of PSD-95. While inhibiting PSD95/nNOS interaction, the nNOS\(_{1-300}\) protein fragment preserves the NMDAR/PSD-95 (PDZ1) interaction, therefore avoiding disruption to additional functions of NMDARs (Cao et al., 2005). Importantly, the nNOS\(_{1-300}\) fragment prevented the induction of stress-activated protein kinase p38 and neuronal death caused by glutamate (Cao et al., 2005). A recent study has also shown that an NOS\(_{1-299}\) fragment inhibits in vitro binding of nNOS to PSD-95 with an IC\(_{50}\) similar of that of Tat-NR2B9c (300nM). When injected intrathecally, Tat-nNOS\(_{1-299}\) inhibited acute thermal hyperalgesia and chronic mechanical allodynia in a rodent model of nociception (Florio et al., 2009).

While biologically active peptides are good tools to investigate protein-protein interactions, the peptidic nature of these competitive peptides may limit potency in a cellular context and more so in vivo, because of rapid degradation and poor pharmacokinetic properties (Dev, 2004). Methods such as viral delivery and
peptide formulations represent approaches to deliver biologically active peptides, however the use of small molecules still represents an optimal option in a clinical setting. Recently, a lentivirus method has been used to overexpress an nNOS1-133 protein fragment containing the PDZ domain and β-finger of nNOS. The stereotactic injection of this nNOS1-133 lentivirus into the right cortex of MCAO mice reduced the infarct area and improved neurological scores when compared to non-treated MCAO mice (Zhou et al., 2010). Significantly, two compounds have been made that bind to the internal PDZ motif of nNOS and inhibit its interaction with PSD-95 (Florio et al., 2009, Zhou et al., 2010), indicating the drugability of PSD-95/nNOS interactions. In the first of these studies, a small-molecule inhibitor, IC87201 (2-((1H-benzo[d][1,2,3]triazol-5-ylamino)methyl)-4,6-dichlorophenol), was identified in a high throughput screen and found to disrupt the PSD-95/nNOS interaction with an IC₅₀ of 31µM. While the binding site of IC87201 has not been identified, in an NMDA-induced thermal hyperalgesia mouse model, intrathecal administration of this compound at a dose of 1pmol had antinociceptive effects. IC87201 was also effective after intraperitoneal injection, suggesting that it crosses the blood brain barrier (Florio et al., 2009). The low IC₅₀ of IC87201 (31µM) has been further improved in a second study, which has identified ZL006 (5-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid), a molecule structurally related to IC87201 (Figure 5). The compound ZL006 has been shown to inhibit NMDAR-dependent NO synthesis in cortical neurons with an IC₅₀ of 82nM and to cross the blood brain barrier after systemic administration. ZL006 is thought to bind the β-finger of nNOS by forming an ionic bond between its carboxyl group and the amino group in Arg-121 of the nNOS PDZ domain and a hydrophobic bond between the hydrophobic ring of ZL006 and Leu-107 or Phe-111 of nNOS, thus hindering the conformational change of nNOS PDZ domain (Zhou et al., 2010). In a MCAO model of stroke, mice treated intravenously with ZL006 at 1.5mg/kg 1h after reperfusion showed improvement in neurological scores and a reduction of infarct size when compared to non-treated mice. Importantly, these results are similar to those found for the lentivirus nNOS1-133 (Zhou et al., 2010). Furthermore, ZL006 did not inhibit nNOS catalytic activity and NMDAR function, did not impair spatial memory and had no effect on aggressive behavior. Taken together, the data suggests that ZL006 may be used to treat stroke without any major side effects (Zhou et al., 2010) and supports the notion that the PSD-95/nNOS interaction is an important target for drug development.

6. Conclusive remarks

The initial discovery of the PSD-95/nNOS interaction was made over a decade ago. Since then this protein complex has been shown to play a key role in the molecular events underlying synaptic plasticity and in the pathophysiology of CNS disorders, such as stroke, hyperalgesia and allodynia. A number of studies have also been aimed at developing agents to alter this PSD-95/nNOS interaction. In summary, disruption of the PSD-95/nNOS interaction has been achieved with both peptide fragments encoding the nNOS PDZ domain and the internal PDZ motif (nNOS1-133 or nNOS1-300) and with small-molecule inhibitors (IC87201 or ZL006). The compounds IC87201 and more so ZL006 have attractive properties to investigate the role of the PSD-95/nNOS interaction in major depressive disorder. Such lead compounds offer the additional advantage of being potential drug candidates for the treatment of depression. In closing, we believe that the uncoupling of nNOS from the NMDA receptor, by regulating the PSD-95/nNOS interaction, may alter glutamate neuronal...
signalling and produce a stress-resilient or antidepressant phenotype, with rapid amelioration in depressive symptoms and a limited side-effect profile.

Acknowledgments
MVD is a Trinity College PhD Scholar. This work is supported by the Department of Physiology and the School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin. We acknowledge grant support from the Health Research Board of Ireland and Science Foundation Ireland.
Figure 1: General structure of the NMDAR/PSD-95/nNOS complex and targets for drug discovery
The ct of NR2A or NR2B contains a PDZ motif (-ESDV) that bind PDZ1 or PDZ2 of PSD-95. In addition, PSD-95 (PDZ2) also interacts with an internal PDZ motif of nNOS (β-finger), at residues 108-111 (-ETTF-). Taken together, these PDZ-based interactions create a ternary complex composed of NR2B (-ESDV)/PSD-95 (PDZ1) and PSD-95 (PDZ2)/nNOS (-ETTF-) interactions. Upon activation of the NMDAR, activation of nNOS triggers the production of nitric oxide and subsequent activation of an intracellular signaling cascade. Over-activation of the NMDA receptor results in an excessive production of nitric oxide, reactive oxygen species (ROS) and peroxynitrites (ONOO-) in addition to the activation of pathways involved in oxidative stress-mediated damage, such as p38 MAPK. Therapeutic strategies to dampen NMDAR activity include disruption of the receptor itself (1) with NMDA receptor antagonists, such as ketamine. Psychosis and psychomotor stimulation preclude their use in clinical practice. Ca^{2+} blockers (2) decrease the overload of Ca^{2+} following opening of the receptor channel but failed to protect neurons from excitotoxicity. Disruption of the NMDAR/PSD-95 interaction with a blocking peptide (Tat-NR2B9c) (3) was shown to have a protective effect in a middle cerebral artery occlusion (MCAO) model of stroke. Effects of this blocking peptide led to further interest in blocking peptides and compounds that disrupt the PSD-95/nNOS interface (4). Pharmacological intervention with NOS inhibitors (5) is an alternative strategy and efforts have been made to synthesise highly selective nNOS inhibitors. **Abbreviations:** L-arg: L-arginine, GC: guanylate cyclase, GK: guanylate kinase, cGMP: cyclic guanosine monophosphate, GTP: guanosine triphosphate, nNOS: neuronal nitric oxide synthase, NO: nitric oxide, p38 MAPK: p38 mitogen activated protein kinase, PDE: phosphodiesterase, PDS-95: postsynaptic density 95, PDZ: PSD-95/discs large/zona occludens, PKG: protein kinase G, ROS: reactive oxygen species, SH3: Src homology 3.

Figure 2: Postsynaptic density proteins
Four postsynaptic density (PSD) family members have been identified. They are classified according to their molecular weight, and include PSD-95 (SAP-90), PSD-93 (Chapsyn-110), synaptic associated proteins 97 kDa and 102 kDa (SAP-97 and SAP-102, respectively). PSD-95 and PSD-93 form multimers, mediated by N-terminal "head to head" interactions. PSD proteins contain three PDZ (PSD-95/DlgA/Zo-1) domains, a SH3 (Src homology 3) domain and a GK (guanylate kinase) domain, which has lost its catalytic activity. SAP-97 additionally contains a L27 domain located at its N-terminus. The longest human isoforms of PSD-95, SAP-102, SAP-97 and PSD-93 are depicted on this figure (accession numbers NM_001365, NM_021120, NM_004087 and NM_001142699 respectively).

Figure 3: Nitric oxide synthases
**A.** Nitric oxide synthases are subdivided into neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) isoforms. Dimerisation creates high-affinity binding sites for L-arginine (Arg), haem and tetrahydrobiopterin (BH4), which are necessary for NOS activity (Zhou and Zhu, 2009). In addition, NOS contains nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactor binding sites, similar to cytochrome P450 reductase (Hobbs et al., 1999). Electrons donated by NADPH transfer from the reductase domain to the oxygenase domain via FAD, FMN and calmodulin (CaM) (Alderton et al., 2001, Zhou and Zhu, 2009). The CaM binding site regulates the electron flow between the 2 regions in a “switch” manner (Su et al., 1995, Hobbs et al., 1999). In the presence of Ca^{2+}, calmodulin associates to Ca^{2+} and forms a Ca^{2+}/CaM complex (Bredt and Snyder, 1990, Su et al., 1995, resulting in electron flow and oxidation of L-arginine to form L-citrulline and nitric oxide (NO) (Zhou and Zhu, 2009). NO activates a soluble guanylate cyclase with an EC_{50} of 1-4 nM (Hall and Attwell, 2008) triggering the formation of cGMP from GTP (Hobbs et al., 1999, Hall and Attwell, 2008) and subsequent activation of protein kinases (PKG I and II) and phosphodiesterases (PDE III) (Guix et al., 2005). **B.** Nitric oxide synthases are products of different genes, display distinct localisations, catalytic properties and inhibitor sensitivity but derive from a common NOS ancestral gene (Alderton et al., 2001). At the amino acid level, nNOS is highly homologous...
among species. In particular, the PDZ domain (residues 1-99) and internal PDZ motif (-ETTF-) of the β-finger (residues 100-130) are conserved between human (Homo sapiens), rat (Rattus norvegicus), mouse (Mus musculus), rabbit (Oryctolagus cuniculus), dog (Canis lupus familiaris), frog (Xenopus laevis) and zebrafish (Danio rerio). A Drosophila nNOS orthologue has been identified and shares 42% homology with its human counterpart, suggesting an important function for nNOS also in Drosophila. Interestingly, Drosophila nNOS seems to lack its internal PDZ motif, questioning the possibility of its interaction with PSD-95. Residues similar between Homo sapiens and Drosophila melanogaster are highlighted in red.

Figure 4: The NMDAR/PSD-95/nNOS Protein Interaction Network

Activation of the NMDA receptor leads to Ca$^{2+}$ influx as well as regulation of other signaling pathways including nNOS. The activation of nNOS via NMDA receptors requires interaction with the scaffold protein PSD-95, which forms an NMDAR/PSD-95/nNOS complex. A. The scaffold protein PSD-95 interacts with more than 40 proteins that can be divided in four categories: receptors, ion channels/pumps, transmembrane proteins and cytosolic proteins. B. The PDZ motif localised at the ct of NR1 or NR2 subunits binds to several cytoskeletal, intracellular signaling, scaffold/adaptor and transmembrane proteins that contain PDZ domains. Specifically, the ct of NMDAR interacts with PDZ2 of PSD-95. C. nNOS contains two non-overlapping binding sites: (i) a canonical PDZ domain (residues 1-99) that binds PDZ motifs and (ii) an “internal” PDZ motif (residues 100-130) that binds PDZ domains of other proteins, such as the PDZ2 of PSD-95. Levels of NO are regulated by proteins interacting with the oxygenase or Ca$^{2+}$/CaM domains of nNOS, such as the Hsp90 (Bender et al., 1999, Peng et al., 2009), NOSIP (Dreyer et al., 2004), PIN (Jaffrey and Snyder, 1996, Tochio et al., 1998) and CaMKIIa (Komeima et al., 2000, Watanabe et al., 2003).

Figure 5: Compounds disrupting the PSD-95/nNOS interaction

Two compounds that disrupt the PSD-95/nNOS interaction have been identified (Florio et al., 2009, Zhou et al., 2010). A. IC87201 (2-(((1H-benzo[d][1,2,3]triazol-5-ylamino)methyl)-4,6-dichlorophenol) disrupts the PSD-95/nNOS interaction with an $IC_{50}$ of 31µM. In mice, this compound was effective in tests of nociception after intraperitoneal injection, with an $EC_{50}$ of 0.1mg/kg. In rats, IC87201 abolished mechanical allodynia when administered intrathecally (at 50 and 100 nmol doses) or intraperitoneally (2mg/kg), suggesting that IC87201 crosses the blood brain barrier. The low $IC_{50}$ of IC87201 (31µM) has been further improved in a second study. B. ZL006 (5-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid) is a molecule structurally related to IC87201, which contains a hydrophobic ring, a hydrophilic ring with a carboxyl group and a linker between the rings that enhance the flexibility of the compound. ZL006 inhibits NMDAR-dependent NO synthesis in cortical neurons with an $IC_{50}$ of 82nM and crosses the blood brain barrier after systemic administration. ZL006 is thought to bind the β-finger of nNOS by forming an ionic bond between its carboxyl group and the amino group in Arg-121 of the nNOS PDZ domain and a hydrophobic bond between the hydrophobic ring of ZL006 and Leu-107 or Phe-111 of nNOS, thus hindering the conformational change of nNOS PDZ domain.
Table 1: Pharmacological approaches to the treatment of major depressive disorder

### 1. Reuptake inhibitors

<table>
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<th>Trade names</th>
<th>Approval</th>
<th>Notes</th>
</tr>
</thead>
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<td><strong>Selective serotonin-reuptake inhibitors (SSRIs)</strong></td>
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<td>- Fluoxetine</td>
<td>Prozac</td>
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</tr>
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<td>✔</td>
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<td>Lustral, Zoloft</td>
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<td>- Fluvoxamine</td>
<td>Faverin, Floxyfral, Luvox</td>
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<td>Cipramil, Celexa, Seroxat</td>
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### 2. MAOIs

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<td>Norset, Remeron, Zispin</td>
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### 4. Adjunctive or augmenting agents

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Table 2: NMDAR/PSD-95/nNOS abnormalities in depression

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n.s.: non significant, ↑: increased, ↓: decreased when compared to matched controls.

Bibliography


KRISTIANSEN, L. V. & MEADOR-WOODRUFF, J. H. 2005. Abnormal striatal expression of transcripts encoding NMDA interacting PSD proteins in schizophrenia, bipolar disorder and major depression. *Schizophr Res*, 78, 87-93.


Fig. 1
Fig. 2
Fig. 3
Fig. 4