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Alterations in NMDA Receptor Subunit Densities and Ligand Binding to Glycine Recognition Sites are Associated with Chronic Anxiety in Alzheimer's Disease

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Abstract

Glutamatergic deficits are established neuropathological features of Alzheimer's disease (AD) and are known to correlate with cognitive impairments. In contrast, the role of glutamatergic alterations in behavioral and psychological symptoms of dementia (BPSD) is unclear. There is considerable preclinical evidence for the importance of glycine recognition sites (GlyRS) of N-methyl-D-aspartate (NMDA) receptors in the regulation of anxiety behaviors. This study aimed to correlate several glutamatergic measures with chronic anxiety in AD. 21 AD patients assessed by the Neuropsychiatric Inventory (NPI) were divided into low anxiety (LA) and high anxiety (HA) subgroups. GlyRS and NMDA channel were measured by brain homogenate binding with [³H]MDL105,519 and [³H]MK-801, respectively. Densities of NMDA receptor NR2A, NR2B and alternate spliced NR1 subunits were quantified by immunoblotting. We found that the binding affinity to GlyRS was significantly higher in HA compared to LA, and this higher GlyRS affinity correlated with selective reduction of NR2A density as well as with elevated anxiety scores. Our observations suggest a novel mechanism whereby subunit specific changes in the NMDA receptor complex may be linked to chronic anxiety in AD via effects on GlyRS function. We propose that NR2A and GlyRS should be further assessed as novel targets of behavioral pharmacotherapy in AD.

1. Introduction

Although Alzheimer's disease (AD) is defined by cognitive impairments, a salient clinical feature of the disease is the occurrence of non-cognitive, neuropsychiatric behaviors such as

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depression, agitation, psychosis and anxiety [18,35]. These behavioral and psychological symptoms of dementia (BPSD)[23] cause significant distress to caregivers and are important factors leading to patient institutionalization [15]. Previous studies which investigated the neurochemical basis of BPSD in AD have focused on perturbations in the cholinergic, serotonergic and catecholaminergic systems [6,26,27,31,52,60]. In contrast, there are few studies on the effects of glutamatergic changes on behavioral symptoms in AD.

Glutamate is the major excitatory neurotransmitter in the mammalian brain whose transmission is mediated by metabotropic as well as ionotropic glutamate receptors (iGluRs). Of the iGluRs, the *N*-methyl-D-aspartate (NMDA) receptors are critically involved in learning and behavioral processes [39,44], and are formed from heterotetrameric complexes of one obligatory NR1 subunit and at least one of the NR2A-D subunits [10,17]. The resultant channel pores can be labeled by non-competitive NMDA antagonists such as dizocilpine (MK-801), which binds to sites within the pore and therefore is not displaced by agonist-site ligands [59]. In the neocortex, NR2A and NR2B are the predominant subunits expressed, while NR2C and NR2D are mainly localized in brainstem and cerebellar regions [50,58]. Upon activation, NMDA receptors mediate calcium ion influx when the Mg^{2+} block is released by membrane depolarization [32]. Interestingly, activation of NMDA receptors requires binding by both glutamate and a co-agonist, glycine [54]. The glycine recognition site (GlyRS) is localized to the NR1 subunit [16,57]; however, binding affinities of ligands for GlyRS seem partly determined by the type of NR2 subunits forming the functional NMDA receptor. For example, NR2B-containing receptors show higher affinity for glycine as well as the GlyRS antagonist (*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1 *H*-indole-2-carboxylic acid (MDL105,519) than NR2A-containing receptors [25,28,30,41], presumably due to subunit differences in steric effects [41].

In contrast to the NR2 subunits, NR1 is normally expressed in excess in the endoplasmic reticulum, where it would be degraded if not assembled with NR2 subunits into functional NMDA receptors [20]. Furthermore, the NR1 gene can give rise to eight different isoforms by alternate splicing of one N-terminal (N1) and two C-terminal (C1 and C2) exons (61). The type of NR1 isoform present determines cellular trafficking and surface expression of the receptor complex, as well as its interaction with cytoskeletal and scaffolding proteins. For example, the exclusion of the C2 exon generates a shorter C-terminus, C2', which contains a PDZ-interacting domain [49]. C2 to C2' switching leads to acceleration of NMDA receptor forward trafficking and increased cell surface expression [38,49].

The glutamatergic system is known to be profoundly affected in AD. Besides widespread losses of glutamatergic pyramidal neurons in the cortical mantle [13], work done in our laboratory and others have also uncovered decreases in neocortical GlyRS binding and glycine mediated potentiation of NMDA channel opening, together with changes in NR1 and NR2 mRNA and protein expression [21,22,42,43,55]. Because glutamatergic dysfunction is implicated in failure of memory consolidation as well as in neuropsychiatric conditions such as schizophrenia and anxiety disorders [13,40,46], it is reasonable to postulate that glutamatergic perturbations may underlie some of the clinical features of AD. Indeed, glutamatergic dysfunction, along with cholinergic deficits, strongly correlated with cognitive decline [12]. However, within the AD group, it is not known whether glutamatergic changes are related to BPSD. Preclinical and animal studies have suggested that GlyRS function is particularly relevant to anxiety-related behaviors, with agonists displaying anxiogenic properties, whilst antagonists are often anxiolytic [1,2,29,45]. In humans, the orbitofrontal cortex is highly susceptible to AD pathology, and many non-cognitive behaviors are likely due to disruption of widespread cortical and subcortical neural systems that involve this region [56]. In order to test the hypothesis that alterations of glutamatergic neurochemistry underlie anxiety symptoms in AD, we measured NMDA GlyRS and open channel binding to radioligands, as well as protein levels

of NR2A, NR2B, NR1, and two alternative spliced forms at the C2 exon of NR1, namely NR1^{C2} and NR1^{C2'}, in the postmortem orbitofrontal cortex of a cohort of behaviorally assessed AD patients.

2. Materials and methods

2.1. Patients, clinical and neuropathologic assessments

The cohort of patients with a diagnosis of definite AD [33] from which subjects in this study were derived has been described previously [53]. In brief, subjects were recruited and assessed at the University of California, Los Angeles Alzheimer Disease Research Center (UCLA-ADRC) after approval by the UCLA Medical Institutional Review Board. Duration of dementia, severity of dementia (measured with the Mini-Mental State Examination [11]), Activities of Daily Living (ADL) ratings [14], drug histories and other clinical information were obtained from the UCLA-ADRC longitudinal study database and from patient charts. None of the subjects were on memantine medication. Behavioral symptoms in the subjects were assessed by the Neuropsychiatric Inventory (NPI, [8]), a caregiver directed semi-structured interview which scores each of ten behaviors on a 12-point scale based on both frequency (1 to 4) and severity (1 to 3) of behavioral change. Caregivers were interviewed by telephone, which had previously been established as a reliable means of obtaining NPI information [8]. Caregivers were asked to rate the subjects' behaviors both in the year prior to death (acute) as well as over the entire disease course (chronic). Scores for chronic, rather than acute, behaviors were considered to be of greatest relevance because the former are more likely to be reflective of long term, 'trait' changes, rather than arising from agonal states. Therefore, based on our *a priori* hypothesis, we selected from among the available subjects two patient subgroups with similar behavioral profiles (see Figure 1) except for chronic anxiety, which were then designated as the no- or low anxiety (LA, NPI chronic anxiety composite scores 0–1, n = 10) and high anxiety (HA, NPI chronic anxiety scores ≥ 2 , n = 11) subgroups. At death, brains were removed from subjects with postmortem delays < 48 hours, and AD diagnoses were neuropathologically confirmed in all subjects using the Consortium to Establish a Registry for AD (CERAD) criteria (36). Staging of the severity of AD neuropathology [5] was also performed.

2.2. Postmortem Tissue Processing

All chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich Ltd. unless otherwise stated. After removal of brain, portions were fixed and used for neuropathologic assessments (see above) while gray matter from the orbitofrontal gyrus (Brodmann area 11) was dissected and fresh frozen at -75°C . When ready to process, the brain fragments were thawed on ice, dissected free of meninges, and homogenized with an Ultra-Turrax homogenizer (IKA Labortechnik, 15 s maximum setting) in Tris-HCl buffer (50 mM, pH 7.7) followed by two washes with Tris-HCl buffer to obtain crude brain homogenates (final concentration 50–100 mg tissue wet weight / ml) for subsequent neurochemical assays. Alternatively, tissues were homogenized in 10 mM sodium phosphate buffer, pH 7.4 containing 1.0 mM EDTA and protease inhibitors (Complete Mini, Roche Diagnostics) followed by diluting 1:1 and boiling in Laemmli sample buffer (Bio-Rad Laboratories) for immunoblotting experiments. Not all cases were available for all assays.

2.3. Radioligand Binding Assays

Measurement of the NMDA receptor GlyRS and open channel were performed by homogenate binding to [³H]MDL105,519 (specific activity 71.0 Ci/mmol) and [³H]MK-801 (sp. act. 28.9 Ci/mmol, both from Perkin Elmer Life Sciences USA), respectively. Aliquots of brain membrane homogenates were incubated in triplicates with six or seven concentrations of [³H]MDL105,519 (0.5–20 nM) in a total volume of 0.5 mL HEPES buffer, pH 7.4 for 30 min at

4°C. 1 mM unlabelled glycine was added to parallel series of binding assays to define non-specific binding. Incubation was terminated by vacuum filtration onto polyethylenimine-treated Whatman GF/B filters (Whatman plc) using a Skatron cell harvester (Molecular Diagnostics Inc.) followed by washing with ice-cold sodium phosphate buffer. Filters were then dried and punched into vials, and aliquots of scintillant (Optiphase HiSafe 2, Perkin Elmer Life Sciences) were added for measurement of bound radioactivity by liquid scintillation spectrophotometry using a Wallac beta counter. Assays for [³H]MK-801 binding (0.5 – 30 nM, 60 min at room temperature) were as above, except that brain homogenates were preincubated in 50 mM Tris-HCl buffer, pH 7.7 for 60 min at 30°C to facilitate degradation of endogenous glutamate. To ensure maximal stimulation and channel opening, [³H]MK-801 binding was performed in 50 mM Tris-HCl buffer, pH 7.7, containing 250 μM spermine, 25 μM glycine, and 20 μM L-glutamate [37]. Nonspecific binding was determined by adding 10 μM unlabelled MK-801 maleate. Values of binding affinity (K_D , in nM) and binding density (B_{max} , in fmol/mg protein) were derived from binding data by Scatchard analyses followed by iterative curve-fitting [34]. Binding data fitted with one-site binding with Hill constants around 1.0. All neurochemical assays were performed blind to clinical information.

2.4. Immunoblotting

Primary antibodies used in this study were anti-NR1, anti-NR2B (rabbit polyclonals from Chemicon Inc.), anti-NR2A (mouse monoclonal from Chemicon Inc.), anti-NR1^{C2}, anti-NR1^{C2'} (rabbit polyclonals from Upstate Biotechnology), and anti-β-actin (mouse monoclonal from Sigma-Aldrich Ltd.). Boiled brain homogenates in Laemmli sample buffer were electrophoretically separated on 7 or 10% polyacrylamide gels, transferred onto nitrocellulose membranes, and blocked in 10 mM phosphate buffered saline, pH 7.4, 0.1% Tween 20 (PBST) / 5% skim milk for 1 h before immunoblotting with primary antibody (1:1000 dilution, except β-actin at 1:5,000 dilution) in PBST / 1% milk overnight at 4°C or 3 h at room temperature. Following washings in PBST / 1% milk and incubation with horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse antibody (1:10,000, Jackson ImmunoResearch Inc.), immunoreactive bands on the membranes were visualized by enhanced chemiluminescence and quantified by image analyzer (UVItec Ltd.). Membranes were then stripped and reblotted with anti-β-actin to control for sample loading across lanes. One lane of external standard consisting of known amounts of protein from a single homogenate sample were loaded for each membrane for normalization of data. Normalized immunoblot optical densities are expressed in arbitrary units. Exposure times for chemiluminescence detection were adjusted to ensure none of the bands reached saturation.

2.5. Statistical analyses

All statistical analyses were performed using the SPSS 11.0 for Windows software (SPSS Inc.). Comparisons of demographic and neurochemical variables between LA and HA groups were performed by Student's *t*-tests, while immunoblot densities were compared by Mann-Whitney tests. Non-parametric tests were also used for comparisons and correlations involving immunoblot densities or NPI scores, because multiplication of the frequency (1 to 4) and severity (1 to 3) sub-scores precludes composite scores of 5, 7, 10 or 11, resulting in non-normal distribution of NPI data. For all analyses, the null hypothesis was rejected at $p < 0.05$.

3. Results

3.1. Demographic, Disease and Neurochemical Variables in Chronic Anxiety Subgroups of AD

Demographic and disease factors including age, post-mortem interval, duration of dementia, predeath MMSE and ADL scores, as well as distribution of Braak staging appeared to be matched between the LA and HA groups (Table 1), although there was a non-significant trend

towards higher total NPI scores in the HA subgroup. Furthermore, except for the behavior under study (anxiety), the chronic NPI composite scores for the other nine behaviors were not significantly different between the LA and HA groups (Figure 1A). There was no significant difference between LA and HA subgroups in any behavior for acute NPI scores (Mann-Whitney $p > 0.05$, data not shown). For the neurochemical measures, both K_D and B_{max} values for [^3H]MK-801 binding in the orbitofrontal cortex were unchanged in HA compared to LA, while K_D for [^3H]MDL105,519 was significantly reduced in HA (Table 1).

3.2. Correlations of Neurochemical Variables, Immunoblot Densities, and Chronic Anxiety in AD

Figures 1B and C show that [^3H]MDL105,519 K_D , but not B_{max} , was negatively correlated with chronic anxiety scores. Figure 2 shows that immunoblot densities of NMDA NR1 subunit (total and alternate spliced), and the NR1^{C2} to NR1^{C2'} ratio were not significantly different between LA and HA subgroups (Figure 2B). NR1^{C2} : NR1^{C2'} values also did not correlate with either chronic anxiety scores or with [^3H]MDL105,519 K_D (Figure 2C). In contrast, immunoblot densities for NR2A subunit, as well as the NR2A to NR2B ratio, were significantly reduced in HA, while NR2B appeared unchanged (Figure 3B). Furthermore, NR2A : NR2B negatively correlated with chronic anxiety scores, and positively correlated with [^3H]MDL105,519 K_D (Figure 3C). Similar results were also obtained when NR2A alone was correlated with chronic anxiety scores and [^3H]MDL105,519 K_D (Spearman $p < 0.05$, data not shown). The current data for changes of NR2A : NR2B in anxiety subgroups indicate a selective reduction of NR2A subunits relative to NR2B, since non-selective losses of NMDA receptor populations would have resulted in decreases in both NR2 subunits, while NR2A:NR2B would remain unchanged.

4. Discussion

In the present study, we investigated the glutamatergic correlates of anxiety in AD by defining, from a relatively small but well-characterized cohort, two patient subgroups with similar demographic and disease indices (see Table 1) as well as similar behavioral profiles except for anxiety (see Figure 1A). This helped avoid potential confounding from co-morbid behaviors or from unexpected effects of demographic and disease variables on neurochemical measures. We then compared several glutamatergic markers in the orbitofrontal cortex of the anxiety subgroups, and report here that relative to the low anxiety (LA) subgroup, the high anxiety (HA) subgroup showed higher affinity of binding [^3H]MDL105,519 by GlyRS (as denoted by decreased K_D values, Table 1). In support of this finding, we further showed that [^3H]MDL105,519 K_D values for all available cases correlated negatively with NPI chronic anxiety scores, such that those patients with high anxiety scores generally displayed high affinity (i.e., low K_D) for [^3H]MDL105,519. Next, we showed that of the various NMDA receptor subunits and splice variants measured with immunoblotting (NR1, NR1^{C2}, NR1^{C2'}, NR2A and NR2B), only NR2A densities were reduced in the HA subgroup compared with LA subgroup. This selective decrease in NR2A (expressed as NR2A:NR2B) also correlated with increased anxiety scores as well as increased binding affinity for MDL105,519. Given that MDL105,519 shows higher affinity for NR1/NR2B than for NR1/NR2A receptors [30], apparent GlyRS affinity may increase with proportional down-regulation of lower affinity, NR2A-containing receptor pools, and this change in affinity may in turn underlie anxiety in AD.

Our findings suggest a neurochemical basis for the development of anxiety in AD, and raise several issues which merit further discussion. It is interesting to speculate on the possible pathogenic mechanisms underlying anxiety symptoms in patients with altered GlyRS. Although we did not examine the properties of GlyRS binding to the endogenous agonist glycine in the AD subjects, there is evidence that both glycine and MDL105,519 show higher

affinity for NR1/NR2B compared with NR1/NR2A [25,28,30,41]. Therefore, the increased GlyRS binding affinities in these subjects, putatively as a result of NR2A loss, may lead to GlyRS hypersensitivity to endogenous levels of amino acid agonists. Higher GlyRS affinities may also reduce the glycine-sensitive desensitization of NMDA receptors normally observed after receptor activation by glutamate and glycine [54]. Both of these processes may lead to GlyRS hyperfunction and resultant anxiogenic effects. However, assessment of the validity of our postulate requires further consideration of whether GlyRS are normally saturated. Previous studies have reported increased or unchanged levels of glycine in AD brain homogenates [19,47], but glycine concentrations in the synaptic clefts are not known. Adding to the complexity of this question is the unclear status of synaptic glycine transporters which tightly regulate extracellular glycine [48]. Importantly, our postulate would imply that GlyRS antagonists may be efficacious for the treatment of anxiety in some AD patients. However, more work is needed in the development of compounds with suitable pharmacological profiles so that anxiolysis can be achieved without worsening NMDA receptor function or memory impairment [7,9].

Studies which have investigated changes of individual NR subunits in AD compared to controls often reported concomitant reductions of NR1, NR2A and NR2B subunits which correlated with loss of synaptic proteins [3,21,51], indicative of non-specific ablation of NMDA receptors through neuronal degeneration. In contrast, mechanisms underlying a further, NR2A-selective reduction in the high-anxiety AD subgroup as reported here are unclear. The lower NR2A did not seem to affect densities of total NMDA receptors (as labeled by the non-NR2A/2B selective open channel ligand, [³H]MK-801 [28]), which suggests higher proportions of NR2B or other subunits being assembled with NR1 into functional receptors. However, there is at present no evidence for the existence of such compensatory processes. Other possible mechanisms for NR2A changes in HA patients meriting further investigation include degeneration of NR2A-containing neuronal populations, genetic factors (e.g., functional polymorphisms of NR2A gene promoter, GRIN2A [24]) as well as altered phosphorylation status [51]. It is worth noting that our postmortem results seem to contradict recent work showing anxiolytic and antidepressantlike effects in NR2A knockout mice [4]. However, the GlyRS binding status in the knockout mice are unknown, and it is also unclear whether compensatory plasticity in the expression of other NR2 subunits occur in these animals. Furthermore, a complete absence of NR2A in the knockout animals may have pleiotropic behavioral effects which potentially interact, e.g., antidepressant and anxiolysis behaviors [4]; whereas in our study, reduced orbitofrontal NR2A densities and [³H]MDL105,519 binding did not correlate with depression or other behavioral scores except anxiety (Spearman $p > 0.05$, data not shown). Nevertheless, one limitation of this and other correlational studies is the inability to prove causation. Therefore, even though the postulate of NR2A reductions leading to GlyRS changes and anxiety is biologically plausible, one cannot exclude the possibility that the observations of NR2A and GlyRS changes are in response to neurochemical changes in other transmitter systems which also give rise to anxiety. Furthermore, because immunoblotting is unable to distinguish between surface expressed and cytoplasmic pools of NR2A, the functional significance of NR2A losses need to be further clarified.

In contrast to the NR2A, we did not find significant differences in total NR1, NR1^{C2} and NR1^{C2'} expression (p values of 0.11, 0.93, 0.39, respectively) between the anxiety subgroups. There also did not seem to be significant switching of C2 / C2' (expressed by NR1^{C2}:NR1^{C2'}, $p = 0.45$), suggesting that NR1 does not play a significant role in anxiogenesis in AD. However, the correlation between NR1^{C2}:NR1^{C2'} and [³H]MDL105,519 K_D approached significance ($p = 0.054$, see Figure 2C), and further cell or animal based studies may be worthwhile to investigate the possible effects of NR1 gene splicing on GlyRS binding properties.

To conclude, a large number of studies suggest that BPSD in AD arise from multiple independent or interacting neurochemical mechanisms involving perturbations of cholinergic, serotonergic, and several other transmitter systems in the neocortex, but to date there is little data on the possible pathogenic role of glutamatergic changes in these behaviors. Based on the observed correlations of neurochemical measures with behavioral data in a cohort of behaviorally assessed AD patients, we now propose a novel mechanism whereby specific down-regulation of NMDA receptor NR2A subunit may alter the ligand binding properties of GlyRS, leading to increased receptor activation and possibly becoming anxiogenic. Our findings point to NR2A and GlyRS as potential targets of anxiolysis treatment in AD. However, more preclinical work is needed to elucidate the mechanisms of NR2A changes as well as how increased GlyRS activation leads to anxiety symptoms. Further studies on the efficacy and safety of using GlyRS or NMDA receptor subunit-specific ligands in AD behavioral pharmacotherapy are also needed.

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Reference List

1. Anthony EW, Nevins ME. Anxiolytic-like effects of N-methyl-D-aspartate-associated glycine receptor ligands in the rat potentiated startle test. *Eur J Pharmacol* 1993;250:317–324. [PubMed: 7906654]
2. Baron BM, Harrison BL, Kehne JH, Schmidt CJ, van Giersbergen PL, White HS, Siegel BW, Senyah Y, McCloskey TC, Fadayel GM, Taylor VL, Murawsky MK, Nyce P, Salituro FG. Pharmacological characterization of MDL 105,519, an NMDA receptor glycine site antagonist. *Eur J Pharmacol* 1997;323:181–192. [PubMed: 9128837]
3. Bi H, Sze CI. N-methyl--aspartate receptor subunit NR2A and NR2B messenger RNA levels are altered in the hippocampus and entorhinal cortex in Alzheimer's disease. *J Neurol Sci* 2002;200:11–18. [PubMed: 12127670]
4. Boyce-Rustay JM, Holmes A. Genetic Inactivation of the NMDA Receptor NR2A Subunit has Anxiolytic- and Antidepressant-Like Effects in Mice. *Neuropsychopharmacology* 2006;31:2405–2414. [PubMed: 16482087]
5. Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol (Berl)* 1991;82:239–259. [PubMed: 1759558]
6. Chen CP, Alder JT, Bowen DM, Esiri MM, McDonald B, Hope T, Jobst KA, Francis PT. Presynaptic serotonergic markers in community-acquired cases of Alzheimer's disease: correlations with depression and neuroleptic medication. *J Neurochem* 1996;66:1592–1598. [PubMed: 8627315]
7. Chen HS, Lipton SA. The chemical biology of clinically tolerated NMDA receptor antagonists. *J Neurochem* 2006;97:1611–1626. [PubMed: 16805772]
8. Cummings JL, Mega M, Gray K, Rosenberg-Thompson S, Carusi DA, Gornbein J. The Neuropsychiatric Inventory: comprehensive assessment of psychopathology in dementia. *Neurology* 1994;44:2308–2314. [PubMed: 7991117]
9. Danysz W, Parsons CG, Bresink I, Quack G. Glutamate in CNS disorders. *Drug News Perspect* 1996;8:261–277.
10. Dingledine R, Borges K, Bowie D, Traynelis SF. The Glutamate Receptor Ion Channels. *Pharmacol Rev* 1999;51:7–62. [PubMed: 10049997]
11. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975;12:189–198. [PubMed: 1202204]
12. Francis PT. Glutamatergic systems in Alzheimer's disease. *Int J Geriatr Psychiatry* 2003;18:S15–S21. [PubMed: 12973746]

13. Francis PT, Sims NR, Procter AW, Bowen DM. Cortical pyramidal neurone loss may cause glutamatergic hypoactivity and cognitive impairment in Alzheimer's disease: investigative and therapeutic perspectives. *J Neurochem* 1993;60:1589–1604. [PubMed: 8473885]
14. Galasko D, Bennett D, Sano M, Ernesto C, Thomas R, Grundman M, Ferris S. An inventory to assess activities of daily living for clinical trials in Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *Alzheimer Dis Assoc Disord* 1997;11:S33–S39. [PubMed: 9236950]
15. Gilley DW, Bienias JL, Wilson RS, Bennett DA, Beck TL, Evans DA. Influence of behavioral symptoms on rates of institutionalization for persons with Alzheimer's disease. *Psychol Med* 2004;34:1129–1135. [PubMed: 15554582]
16. Hirai H, Kirsch J, Laube B, Betz H, Kuhse J. The glycine binding site of the N-methyl-D-aspartate receptor subunit NR1: Identification of novel determinants of co-agonist potentiation in the extracellular M3-M4 loop region. *Proc Natl Acad Sci USA* 1996;93:6031–6036. [PubMed: 8650214]
17. Hollmann M, Heinemann S. Cloned glutamate receptors. *Annu Rev Neurosci* 1994;17:31–108. [PubMed: 8210177]
18. Hope T, Keene J, Fairburn CG, Jacoby R, McShane R. Natural history of behavioural changes and psychiatric symptoms in Alzheimer's disease. A longitudinal study. *Br J Psychiatry* 1999;174:39–44. [PubMed: 10211149]
19. Hoyer S, Nitsch R. Cerebral excess release of neurotransmitter amino acids subsequent to reduced cerebral glucose metabolism in early-onset dementia of Alzheimer type. *J Neural Transm* 1989;75:227–232. [PubMed: 2926384]
20. Huh KH, Wenthold RJ. Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. *J Biol Chem* 1999;274:151–157. [PubMed: 9867823]
21. Hynd MR, Scott HL, Dodd PR. Differential expression of N-methyl-D-aspartate receptor NR2 isoforms in Alzheimer's disease. *J Neurochem* 2004;90:913–919. [PubMed: 15287897]
22. Hynd MR, Scott HL, Dodd PR. Selective loss of NMDA receptor NR1 subunit isoforms in Alzheimer's disease. *J Neurochem* 2004;89:240–247. [PubMed: 15030408]
23. IPA. Proceedings of an international consensus conference. Lansdowne, Virginia: 1996 Apr. Behavioral and Psychological Signs and Symptoms of Dementia: Implications for Research and Treatment. *Int Psychogeriatr* 1996;8(Suppl 3):215–552.
24. Itokawa M, Yamada K, Yoshitsugu K, Toyota T, Suga T, Ohba H, Watanabe A, Hattori E, Shimizu H, Kumakura T, Ebihara M, Meerabux JM, Toru M, Yoshikawa T. A microsatellite repeat in the promoter of the N-methyl-D-aspartate receptor 2A subunit (GRIN2A) gene suppresses transcriptional activity and correlates with chronic outcome in schizophrenia. *Pharmacogenetics* 2003;13:271–278. [PubMed: 12724619]
25. Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M. Molecular diversity of the NMDA receptor channel. *Nature* 1992;358:36–41. [PubMed: 1377365]
26. Lai MK, Lai OF, Keene J, Esiri MM, Francis PT, Hope T, Chen CP. Psychosis of Alzheimer's disease is associated with elevated muscarinic M₂ binding in the cortex. *Neurology* 2001;57:805–811. [PubMed: 11552008]
27. Lai MK, Tsang SW, Francis PT, Esiri MM, Keene J, Hope T, Chen CP. Reduced serotonin 5-HT_{1A} receptor binding in the temporal cortex correlates with aggressive behavior in Alzheimer disease. *Brain Res* 2003;974:82–87. [PubMed: 12742626]
28. Laurie DJ, Seeburg PH. Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. *Eur J Pharmacol* 1994;268:335–345. [PubMed: 7528680]
29. Leeson PD, Iversen LL. The glycine site on the NMDA receptor: structure-activity relationships and therapeutic potential. *J Med Chem* 1994;37:4053–4067. [PubMed: 7990104]
30. Li Z, Becker J, Noe CR. Functional expression of recombinant N-methyl-D-aspartate receptors in the yeast *Saccharomyces cerevisiae*—localization and pharmacological characterization. *Eur J Biochem* 1998;252(3):391–399. [PubMed: 9546654]
31. Matthews KL, Chen CP, Esiri MM, Keene J, Minger SL, Francis PT. Noradrenergic changes, aggressive behavior, and cognition in patients with dementia. *Biol Psychiatry* 2002;51:407–416. [PubMed: 11904135]

32. Mayer ML, Westbrook GL, Guthrie PB. Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurones. *Nature* 1984;309:261–263. [PubMed: 6325946]
33. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984;34:939–944. [PubMed: 6610841]
34. McPherson GA. Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J Pharmacol Methods* 1985;14:213–228. [PubMed: 3840547]
35. Mega MS, Cummings JL, Fiorello T, Gornbein J. The spectrum of behavioral changes in Alzheimer's disease. *Neurology* 1996;46:130–135. [PubMed: 8559361]
36. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991;41:479–486. [PubMed: 2011243]
37. Mitchell JJ, Anderson KJ. Age-related changes in [^3H]MK-801 binding in the Fischer 344 rat brain. *Neurobiol Aging* 1998;19:259–265. [PubMed: 9662001]
38. Mu Y, Otsuka T, Horton AC, Scott DB, Ehlers MD. Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. *Neuron* 2003;40:581–594. [PubMed: 14642281]
39. Newcomer JW, Krystal JH. NMDA receptor regulation of memory and behavior in humans. *Hippocampus* 2001;11:529–542. [PubMed: 11732706]
40. Olney JW, Newcomer JW, Farber NB. NMDA receptor hypofunction model of schizophrenia. *J Psychiatr Res* 1999;33:523–533. [PubMed: 10628529]
41. Priestley T, Laughton P, Myers J, Le BB, Kerby J, Whiting PJ. Pharmacological properties of recombinant human *N*-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. *Mol Pharmacol* 1995;48:841–848. [PubMed: 7476914]
42. Procter AW, Stirling JM, Stratmann GC, Cross AJ, Bowen DM. Loss of glycine-dependent radioligand binding to the *N*-methyl-D- aspartate-phencyclidine receptor complex in patients with Alzheimer's disease. *Neurosci Lett* 1989;101:62–66. [PubMed: 2570384]
43. Procter AW, Wong EH, Stratmann GC, Lowe SL, Bowen DM. Reduced glycine stimulation of [^3H] MK-801 binding in Alzheimer's disease. *J Neurochem* 1989;53:698–704. [PubMed: 2569500]
44. Riedel G, Platt B, Micheau J. Glutamate receptor function in learning and memory. *Behav Brain Res* 2003;140:1–47. [PubMed: 12644276]
45. Schmitt ML, Coelho W, Lopes-de-Souza AS, Guimaraes FS, Carobrez AP. Anxiogenic-like effect of glycine and D-serine microinjected into dorsal periaqueductal gray matter of rats. *Neurosci Lett* 1995;189:93–96. [PubMed: 7609926]
46. Simon AB, Gorman JM. Advances in the treatment of anxiety: targeting glutamate. *NeuroRx* 2006;3:57–68. [PubMed: 16490413]
47. Smith CC, Bowen DM, Sims NR, Neary D, Davison AN. Amino acid release from biopsy samples of temporal neocortex from patients with Alzheimer's disease. *Brain Res* 1983;264:138–141. [PubMed: 6133580]
48. Smith KE, Borden LA, Hartig PR, Branchek T, Weinshank RL. Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. *Neuron* 1992;8:927–935. [PubMed: 1534013]
49. Standley S, Roche KW, McCallum J, Sans N, Wenthold RJ. PDZ domain suppression of an ER retention signal in NMDA receptor NR1 splice variants. *Neuron* 2000;28(3):887–898. [PubMed: 11163274]
50. Suen PC, Wu K, Xu JL, Lin SY, Levine ES, Black IB. NMDA receptor subunits in the postsynaptic density of rat brain: expression and phosphorylation by endogenous protein kinases. *Mol Brain Res* 1998;59:215–228. [PubMed: 9729394]
51. Sze C, Bi H, Kleinschmidt-DeMasters BK, Filley CM, Martin LJ. *N*-Methyl-D-aspartate receptor subunit proteins and their phosphorylation status are altered selectively in Alzheimer's disease. *J Neurol Sci* 2001;182:151–159. [PubMed: 11137521]

52. Tanaka Y, Meguro K, Yamaguchi S, Ishii H, Watanuki S, Funaki Y, Yamaguchi K, Yamadori A, Iwata R, Itoh M. Decreased striatal D2 receptor density associated with severe behavioral abnormality in Alzheimer's disease. *Ann Nucl Med* 2003;17:567–573. [PubMed: 14651356]
53. Tekin S, Mega MS, Masterman DM, Chow T, Garakian J, Vinters HV, Cummings JL. Orbitofrontal and anterior cingulate cortex neurofibrillary tangle burden is associated with agitation in Alzheimer disease. *Ann Neurol* 2001;49:355–361. [PubMed: 11261510]
54. Thomson AM. Glycine is a coagonist at the NMDA receptor/channel complex. *Prog Neurobiol* 1990;35:53–74. [PubMed: 1699248]
55. Tsang SW, Pomakian J, Marshall GA, Vinters HV, Cummings JL, Chen CP, Wong PT, Lai MK. Disrupted muscarinic M1 receptor signaling correlates with loss of protein kinase C activity and glutamatergic deficit in Alzheimer's disease. *Neurobiol Aging*. 2006In Press:
56. Van Hoesen GW, Parvizi J, Chu CC. Orbitofrontal cortex pathology in Alzheimer's disease. *Cereb Cortex* 2000;10:243–251. [PubMed: 10731219]
57. Wafford KA, Kathoria M, Bain CJ, Marshall G, Le BB, Kemp JA, Whiting PJ. Identification of amino acids in the *N*-methyl-D-aspartate receptor NR1 subunit that contribute to the glycine binding site. *Mol Pharmacol* 1995;47:374–380. [PubMed: 7870047]
58. Wenzel A, Scheurer L, Kunzi R, Fritschy JM, Mohler H, Benke D. Distribution of NMDA receptor subunit proteins NR2A, 2B, 2C and 2D in rat brain. *Neuroreport* 1995;7:45–48. [PubMed: 8742413]
59. Wong EH, Knight AR, Woodruff GN. [³H]MK-801 labels a site on the *N*-methyl-D-aspartate receptor channel complex in rat brain membranes. *J Neurochem* 1988;50:274–281. [PubMed: 2826686]
60. Wynn ZJ, Cummings JL. Cholinesterase inhibitor therapies and neuropsychiatric manifestations of Alzheimer's disease. *Dement Geriatr Cogn Disord* 2004;17:100–108. [PubMed: 14564129]
61. Zukin RS, Bennett MV. Alternatively spliced isoforms of the NMDAR1 receptor subunit. *Trends Neurosci* 1995;18:306–313. [PubMed: 7571011]

Figure 1.

A, NPI (Neuropsychiatric Inventory) chronic mean composite scores for low and high anxiety subgroups of AD. *Significantly different from low anxiety subgroup, $p < 0.01$ (Mann-Whitney). **B, Binding properties of NMDA receptor glycine recognition sites correlate with chronic anxiety in AD.** $[\text{}^3\text{H}]\text{MDL105,519}$ binding affinity (K_D , in nM) and density (B_{max} , in fmol/mg protein) with NPI anxiety composite scores. *Significant Spearman correlation.

Figure 2. Densities of NMDA receptor NR1 subunits (total and alternate spliced) in chronic anxiety subgroups of AD

A, representative immunoblots of NMDA receptor NR1, NR1^{C2} and NR1^{C2'} normalized to β -actin (not shown) with molecular weight markers (in kDa) as indicated. **B**, comparisons of mean normalized immunoblot densities for NR1, NR1^{C2}, NR1^{C2'} and ratio of NR1^{C2} to NR1^{C2'} in high anxiety (HA, n = 8) subgroup expressed as a percentage of the low anxiety (LA, n = 8) subgroup. **C**, correlations of NR1^{C2}:NR1^{C2'} with NPI anxiety scores (top) and [³H]MDL105,519 K_D values (bottom).

Figure 3. Densities of NMDA receptor NR2 subunits in chronic anxiety subgroups of AD

A, representative immunoblots of NMDA receptor NR2A and NR2B subunits normalized to β-actin (bottom lane). **B**, comparisons of mean normalized immunoblot densities for NR2A, NR2B and ratio of NR2A to NR2B in HA (n = 8) expressed as a percentage of LA (n = 8). **C**, correlations of NR2A:NR2B with NPI anxiety scores (top) and [³H]MDL105,519 K_D values (bottom). *Significantly different from LA, $p < 0.05$. **Significant Spearman correlation.

Table 1
Demographic, Disease and Neurochemical Variables in Chronic Anxiety Subgroups of AD

		<i>Low Anxiety (LA)</i> n = 10^a	<i>High Anxiety (HA)</i> n = 11^a
<i>Demographics</i>			
Age at Death (y)		79.6 ± 3	80.1 ± 2
Sex (M/F)		2 / 8	4 / 7
PMI (h)		18.7 ± 4	14.9 ± 3
Education (y)		15.2 ± 0.9	14.2 ± 0.9
<i>Disease Variables</i>			
Duration of dementia (y)		9.2 ± 2	9.7 ± 1
Last MMSE		9.7 ± 4	11.3 ± 2
ADL total score		12.0 ± 2	14.8 ± 0.5
NPI total score		20.4 ± 3	28.7 ± 3
Braak staging	III/IV	1	1
	V/VI	9	10
<i>Neurochemical Variables</i>			
[³ H]MK-801	K _D	6.5 ± 0.7	6.7 ± 0.4
	B _{max}	248 ± 11	238 ± 20
[³ H]MDL105,519	K _D	12.5 ± 1.7	8.2 ± 0.7 [*]
	B _{max}	344 ± 59	220 ± 23

Data are mean ± s.e.m except for sex and Braak staging (in n). ADL, Activities of Daily Living; B_{max}, binding density (in fmol / mg protein); K_D, binding affinity (in nM); MMSE, Mini-Mental State Examination; NPI, Neuropsychiatric Inventory; PMI, post-mortem interval.

^a Maximum number of cases.

^{*} Significantly different from LA, *p* < 0.05 (Student's t-test).