Journal of Neurochemistry, 2006, **98**, 1114–1125 doi:10.1111/j.1471-4159.2006.03954.x

Up-regulation of NMDA receptor subunit and post-synaptic density protein expression in the thalamus of elderly patients with schizophrenia

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Abstract

Numerous studies have described structural and functional abnormalities of the thalamus in schizophrenia, but surprisingly few studies have examined neurochemical abnormalities that accompany these pathological changes. We previously identified abnormalities of multiple molecules associated with glutamatergic neurotransmission, including changes in NMDA receptor subunit transcripts and binding sites and NMDA receptor-associated post-synaptic density (PSD) protein transcripts in the thalamus of elderly patients with schizophrenia. In the present study, we performed western blot analysis to determine whether protein levels of NMDA receptor subunits (NR1, NR2A, NR2B) and associated PSD proteins (NF-L, PSD95, SAP102) are altered in schizophrenia. Thalamic tissue from each subject was grossly dissected into two regions: a dorsomedial region containing limbic-associated dorsomedial, anterior and central medial thalamic nuclei; and a ventral thalamus region that primarily consisted of the ventral lateral nucleus. We observed increased protein expression of the NR2B NMDA receptor subunit and its associated intracellular protein, PSD95, in the dorsomedial thalamus of patients with schizophrenia, but the other molecules were unchanged, and we found no changes in the ventral thalamus. These data provide additional evidence of thalamic neurochemical abnormalities, particularly in thalamic nuclei which project to limbic regions of the brain. Further, these findings provide additional evidence of NMDA receptor alterations in schizophrenia, which may play an important role in the neurobiology of the illness.

Keywords: glutamate, mediodorsal nucleus, NMDA receptor subunit 2B, post-synaptic density protein 95.

J. Neurochem. (2006) **98**, 1114–1125.

The thalamus contains numerous topographically organized nuclei that transmit sensory and higher order inputs to and from cortical and limbic structures (Jones 1998). Through these connections, the thalamus plays a critical role in regulating sensory processing, attention and other executive functions commonly disturbed in schizophrenia. Numerous *in vivo* imaging studies have identified thalamic abnormalities in schizophrenia. These include reports of reduced thalamic volume (Andreasen *et al*. 1990, 1994; Flaum *et al*. 1995; Buchsbaum and Hazlett 1998; Gur *et al*. 1998; Staal *et al*. 1998; Dasari *et al*. 1999; Ettinger *et al*. 2001; Gilbert *et al*. 2001; Konick and Friedman 2001), reduced thalamic metabolism (Resnick *et al*. 1988; Tamminga *et al*. 1992; Silbersweig *et al*. 1995; Vita *et al*. 1995; Andreasen *et al*. 1996; Buchsbaum *et al*. 1996; Heckers *et al*. 1998; Hazlett *et al*. 1999), and sensory processing deficits in patients with schizophrenia that, at least partly, involve thalamic hypofunction (Braus *et al*. 2002; Takahashi *et al*. 2004; Butler *et al*. 2005; Kiehl *et al*. 2005; Symond *et al*. 2005). Many post-mortem stereological studies have found decreased cell numbers in the mediodorsal nucleus of the thalamus (Pakkenberg 1990, 1992, 1993; Danos *et al*. 1998, 2002, 2003; Popken *et al*. 2000; Young *et al*. 2000; Byne *et al*. 2002), although two recent studies were unable to replicate these findings (Cullen *et al*. 2003; Dorph-Petersen

Received December 20, 2005; revised manuscript received March 27, 2006; accepted March 29, 2006.

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Abbreviations used: NF-L, neurofilament light; PBS, phosphate-buffered saline; PSD, post-synaptic density; SAP, synapse-associated protein; TBS, Tris-buffered saline.

et al. 2004). Considering the wealth of evidence for structural pathology and thalamic dysfunction in schizophrenia, we conducted a series of studies to examine possible neurochemical substrates that may be associated with thalamic abnormalities in the illness. Much of this work has focused on glutamatergic abnormalities in the thalamus (for review see Clinton and Meador-Woodruff 2004a), partly because glutamate is a predominant neurotransmitter used in the thalamus and because glutamatergic dysfunction has been hypothesized to be involved in schizophrenia (Goff and Coyle 2001).

The strongest evidence for glutamate dysfunction in schizophrenia is the finding that psychotomimetic agents like phencyclidine and ketamine, which block the NMDA glutamate receptor channel, can trigger a schizophrenia-like syndrome in healthy subjects (Domino and Luby 1981) and exacerbate symptoms in patients with schizophrenia (Lahti *et al*. 1995). Clinical data show that NMDA receptor glycine site agonists (e.g. glycine, D-cycloserine and D-serine), which positively modulate NMDA channel function, significantly improve cognitive deficits and negative symptoms when given in combination with anti-psychotics (Javitt *et al*. 1994; Goff *et al*. 1995; Tsai *et al*. 1998). Finally, post-mortem studies have revealed NMDA receptor abnormalities in limbic structures implicated in schizophrenia, including the prefrontal cortex (Dracheva *et al*. 2001; Toyooka *et al*. 2002; Emamian *et al*. 2004; Mueller and Meador-Woodruff 2004), temporal cortex (Grimwood *et al*. 1999; Nudmamud-Thanoi and Reynolds 2004), hippocampus (Dean *et al*. 1999; Gao *et al*. 2000; Toro and Deakin 2005), substantia nigra (Mueller *et al*. 2004) and thalamus (Ibrahim *et al*. 2000; Clinton *et al*. 2003; Clinton and Meador-Woodruff 2004b). Taken together, these data suggest that hypoglutamatergic neurotransmission plays a role in the pathophysiology of schizophrenia, and that boosting NMDA receptor transmission through various mechanisms may be a promising approach for pharmacotherapy of the illness (Javitt 2004).

Glutamate neurotransmission involves several classes of molecules, including pre- and post-synaptic glutamate receptors and receptor-associated intracellular proteins. The NMDA glutamate receptor consists of several subunits, including the obligate NR1 subunit, and combinations of NR2 subunits A–D (Hollmann and Heinemann 1994). NMDA receptors also interact with intracellular proteins enriched in the post-synaptic density (PSD), which link the receptor to the cytoskeleton, kinases, phosphatases, and other signal transduction or receptor trafficking pathways (Sheng and Pak 2000). Considering the complexity of the glutamate system, it is possible that glutamate dysfunction in schizophrenia may involve abnormalities of the NMDA receptor as well as a disruption of other molecules involved in glutamate neurotransmission. Glutamatergic abnormalities that are hypothesized to be present in schizophrenia could be explained by many single, or combinations of, alternative possibilities, including altered release of glutamate, abnormal expression of NMDA receptor subunits, or reduced capacity of NMDA receptors to activate intracellular signal transduction pathways (Krystal *et al*. 1999).

Several studies from our laboratory have revealed alterations in the expression of glutamate receptors and other molecules involved in glutamatergic neurotransmission in the thalamus of elderly patients with schizophrenia (Ibrahim *et al*. 2000; Richardson-Burns *et al*. 2000; Smith *et al*. 2001a,b; Clinton *et al*. 2003, 2004). We initially found reduced NMDA receptor subunit transcripts NR1 and NR2C, and decreased polyamine and glycine binding sites of the NMDA receptor complex in limbic thalamic nuclei in schizophrenia, but we did not find prominent changes in the expression of AMPA, kainate or metabotropic receptors (Ibrahim *et al*. 2000; Richardson-Burns *et al*. 2000). Subsequent studies revealed altered transcript expression of a set of NMDA receptor-associated intracellular molecules, including post-synaptic density protein 95 (PSD95), synapse-associated protein 102 (SAP102), and neurofilament subunits NF-L and NF-M (Clinton *et al*. 2003, 2004), suggesting that glutamate dysfunction in this illness may not only involve alterations in glutamate receptors themselves, but also changes in intracellular molecules critical for glutamate receptor-associated signal transduction. Finally, although much of our work has focused on post-synaptic glutamate receptors and associated molecules, we have also identified abnormalities of pre-synaptic and glial-associated molecules that participate in glutamate neurotransmission (Smith *et al*. 2001a,b). We reported increased expression of transcripts for one of the vesicular glutamate transporters (VGLUT2) in schizophrenia, which may reflect an increase of glutamate release in brain regions that receive thalamic efferent projections, such as the prefrontal cortex (Smith *et al*. 2001a). The expression of glial-localized Excitatory Amino Acid Transporter (EAAT1) and EAAT2 transcripts was also increased, but the neuronally-expressed EAAT3 and EAAT4 transcripts were unchanged in the thalamus in schizophrenia compared with controls (Smith *et al*. 2001b).

Taken together, this series of *in situ* hybridization studies suggests a significant dysregulation of glutamate neurotransmission in the thalamus in schizophrenia. In the present study, using tissue from the same collection of elderly subjects as used in our earlier work, we have performed western blot analysis to determine whether protein expression of the NMDA subunits and associated PSD molecules is also altered in schizophrenia, and whether the pattern of changes parallels those observed for mRNA expression.

Material and methods

Brain tissue preparation

Fifteen elderly patients with schizophrenia and eight non-psychiatrically ill individuals from the Mount Sinai Medical Center Brain

Table 1 Subject characteristics

	Sex	Age	PMI (min)	pH	Cause of death
Control subjects ($n = 8$)	F	78	545	6.6	Cardiopulmonary failure
	M	96	195	6.7	Cardiopulmonary failure
	F	74	180	6.0	Cardiopulmonary failure
	F	82	340	6.1	Cardiopulmonary arrest
	F	64	1145	6.1	Pulmonary edema
	M	93	1140	6.4	Congestive heart failure
	F	73	203	6.3	Acute myocardial infarction
	F	79	460	6.5	Acute myocardial infarction
Schizophrenia patients ($n = 15$)	M	52	1270	6.3	Cardiopulmonary failure
	M	58	800	6.9	Cardiopulmonary failure
	M	57	820	6.1	Cardiopulmonary arrest
	M	69	820	6.2	Cardiopulmonary failure
	F	83	1225	7.1	Cardiopulmonary arrest, pancreatic cancer
	F	85	320	6.3	Cardiopulmonary arrest
	M	73	475	6.5	Cardiopulmonary failure
	M	66	725	6.5	Acute cardiac failure
	M	76	1270	6.1	Cardiogenic shock
	F	97	555	6.5	Cardiopulmonary arrest
	M	59	381	6.2	Pancreatitis
	M	66	504	6.7	Cardiopulmonary arrest
	F	82	1126	6.6	Cardiopulmonary arrest
	F	79	595	6.8	Cardiac arrest
	M	68	1036	6.6	Cardiopulmonary arrest
Control	2M : 6F	79.9 (64–96)	526 (180–1145)	6.3 (6.0–6.7)	
Schizophrenia	10M : 5F	71.3 (52–97)	795 (320–1170)	6.5 (6.1–7.1)	

Bank were studied (Table 1). Next of kin consent was obtained for each subject. Neither age ($t = 1.65$; d.f. $= 21$; $p = 0.11$), nor post-mortem interval ($t = -1.74$; d.f. $= 21$; $p = 0.10$) nor tissue pH ($t = -1.25$; d.f. $= 21$; $p = 0.23$) were significantly different between diagnostic groups, although there was a trend for differences in sex distribution ($\chi^2 = 3.63$; $p = 0.06$). Each patient and comparison brain underwent neuropathological examination, and Alzheimer's disease and other discernable neuropathological conditions was specifically ruled out in each case. Brains were prepared by slicing the left hemisphere into 0.8–1 cm coronal slabs, which were snap frozen. Blocks containing thalamus were initially cryostat-sectioned for *in situ* hybridization studies, and remaining tissue blocks (approximately 3–4 mm thick) were grossly dissected into dorsomedial and ventral thalamic areas (Fig. 1a). These regions were identified for each subject primarily using the descriptions of thalamic architecture of Jones (Steriade *et al*. 1997), and supplemented by a set of detailed atlases (Hirai and Jones 1989; Mai *et al*. 1997). The dorsomedial dissected region contained portions of the dorsomedial nucleus and, in some cases, the anterior nucleus and central medial nucleus of the thalamus. The dissected ventral region contained portions of the ventral anterior and ventral lateral nuclei of the thalamus.

Frozen dorsomedial and ventral thalamic samples (0.1–0.5 g of tissue) from each patient were homogenized in 10 volumes of ice-cold buffer containing 250 mM sucrose, 10 mM sodium phosphate, 1 mM EDTA and a cocktail of protease inhibitors (which included a broad spectrum of serine, cysteine and metalloproteases, as well as calpains; Roche, Indianapolis, IN, USA), with a pH of 7.4. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockland, MA, USA).

Western blot analysis

Dorsomedial and ventral thalamus tissue homogenates (40 μg per lane) from each patient were denatured in sample loading buffer [10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-β-mercaptoethanol, 63 mM Tris (pH 6.8) and 0.01% bromophenol blue] at 95°C for 4 min. Tissue samples, a pre-stained molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA) and an internal control of standard human thalamus homogenate were loaded onto 1.0 mm thick 7.5% polyacrylamide gels and run for 45 min at 100 V using a Mini-PROTEAN II System (Bio-Rad Laboratories, Hercules, CA, USA). As a single gel could not accommodate all of the samples run in duplicate, protein samples from patients with schizophrenia and control subjects were alternately allocated on two polyacrylamide gels, together with the molecular marker and internal positive control. Separated proteins were transferred to nitrocellulose membranes (Bio-Rad), overnight at 11 V, using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) with transfer buffer (25 mM Tris, 192 mM glycine).

Following the transfer procedure, blots were rinsed in high purity water (15 min) and cut into sections containing the proteins of interest. As there was sufficient separation between some of the protein bands of interest, we were able to obtain data on multiple proteins from each set of gels. For instance, one set of membranes

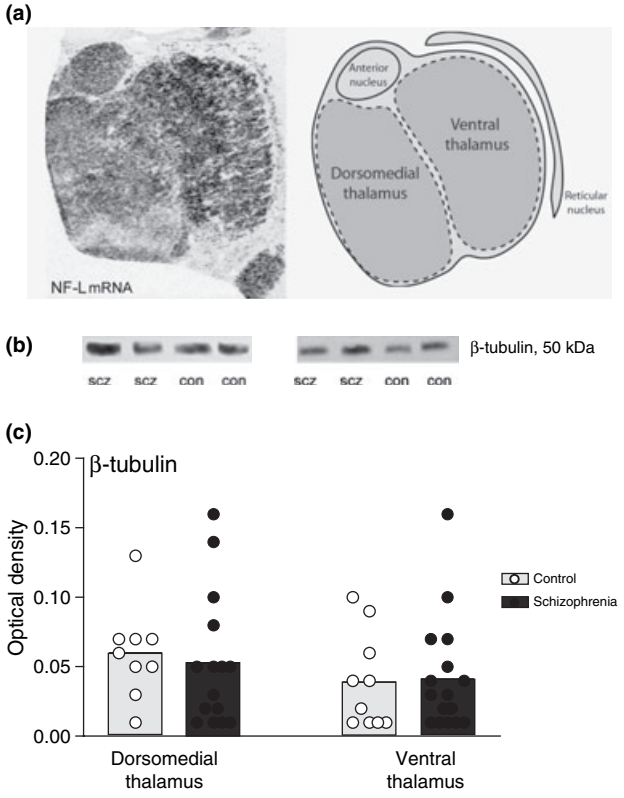

Fig. 1 (a) Thalamic tissue blocks containing thalamus were initially sectioned for *in situ* hybridization, then later dissected for western blot analysis. The top left panel shows a representative section processed for *in situ* hybridization for neurofilament-light (NF-L) mRNA. The top right panel is a schematic diagram of the section, identifying major nuclei present in this section: anterior nucleus, dorsomedial nucleus, ventral thalamus (which includes the ventral anterior, ventral lateral anterior and posterior nuclei) and the reticular nucleus. Thalamic tissue blocks for each subject were dissected into a dorsomedial and ventral portion, outlined by the dashed line in the schematic. Tissue was then weighed, homogenized and prepared for western blot analysis. (b) Representative immunoblots for β-tubulin using dorsomedial and ventral thalamus samples from a patient with schizophrenia (scz) and a control subject (con); lanes show side-by-side duplicates of each subject. (c) Scatterplots represent β-tubulin protein expression levels in the dorsomedial and ventral thalamus in patients with schizophrenia and comparison subjects. β-tubulin protein levels in dorsomedial and ventral thalamus tissue were unchanged between diagnostic groups.

was cut into three portions: the top membrane strip contained the band for 180 kDa NR2B, the middle strip contained the band for 100 kDa NR1 and the bottom strip contained the band for 50 kDa β-tubulin. Three sets of gels were needed to obtain data for all of the NMDA receptor subunits and post-synaptic proteins, and β-tubulin expression was assessed in all gel sets to ensure consistency across the experiments. Membranes were pre-incubated for 20–30 min at room temperature (37°C) in a blocking solution consisting of either 0.1 M phosphate-buffered saline (PBS) or 0.1 M Tris-buffered saline (TBS) (both pH 7.6), 2–5% fat-free dry milk (Carnation, Wilkes-Barre, PA, USA) depending on the antibody and, in some cases, 0.05% Tween-20. Table 2 describes the primary antibodies used in this study, including information regarding the specific blocking solutions and antibody concentrations, which were optimized for each antibody. Membranes were incubated with primary antibodies for the NMDA receptor subunits, associated PSD proteins or β-tubulin in blocking solution overnight at 4°C. Approximately 18 h later, membranes were rinsed twice in distilled water, then incubated for 2 h at room temperature (37°C) with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1 μg/mL; Sigma-Aldrich Corp., St Louis, MO, USA) in the blocking solution indicated in Table 2. Following the 2 h incubation with secondary antibody, membranes were rinsed twice (15 min) in either PBS or TBS (according to the type of blocking solution used), rinsed in either PBS or TBS with 0.05% Tween-20 for 5 min, and finally washed 3–4 times (1 min) with distilled water. Immunolabeling was visualized using an electrochemiluminescence (ECL) western blotting analysis system (Amersham Biosciences, Piscataway, NJ, USA). ECL-treated membranes were placed between two plastic transparencies, then apposed to ECL-Hyperfilm (Amersham Biosciences) for 2–30 min.

Image and data analysis

Densitometry quantification of the band intensities was performed within the range of linear exposure of the film using SCION IMAGE BETA 3B for PC (Scion Corp., Frederick, MD, USA). Duplicate samples were averaged for each subject. All of the blotting was repeated, and differences in either NMDA receptor subunits or PSD proteins were confirmed by an independent set of immunoblotting. In order to control for equal loading, we calculated ratios of the optical density for the antibody of interest to the optical density of the antibody directed against β-tubulin for each patient. Statistical analysis was accomplished with 2-way ANOVA (diagnosis × thalamic subregion), followed by Fisher's post-hoc comparisons. The Shapiro–Wilks' *W*-test was used to ensure normality of all data. Pearson product–moment correlations were used to

Table 2 Immunoblot conditions

Protein	Origin	Corresponding amino acids	Length of peptide	Blocking solution	Dilution	Company
NR1	Rat	917–938	21	5% TBST-milk	1 μg/mL	Upstate Biotechnology
NR2A	Mouse	1241–1441	200	3% PBS-milk	3 μg/mL	Upstate Biotechnology
NR2B	Mouse	1437–1456	19	2% PBS-milk	2 μg/mL	Upstate Biotechnology
NF-L	Human	n.a.	n.a.	3% TBST-milk	0.5 μg/mL	Zymed Laboratories, Inc.
PSD-95	Human	77–299	223	3% PBS-milk	0.05 μg/mL	Upstate Biotechnology
SAP102	Human	93–190	97	3% TBST-milk	10 μg/mL	Chemicon International
β-tubulin	Bovine	412–430	18	3% PBS-milk	0.5 μg/mL	Upstate Biotechnology

determine any possible relationships between continuously distributed variables. Data were analyzed using SPSS 13.0 for Windows, and for all tests $\alpha = 0.05$.

Results

β-tubulin protein expression

We measured the expression of β-tubulin protein in all samples to use as an internal control. The β-tubulin antibody detected a single prominent band at 50 kDa (Fig. 1b). As all of the other proteins of interest run well above this molecular weight, each experimental blot was cut so that the top pieces could be processed to detect the NMDA receptor subunits or associated PSD proteins, and the bottom piece was processed to assess β-tubulin in parallel for each subject. Data for NMDA receptor subunits and associated PSD proteins are presented as a ratio of the optical density value for the protein band of interest over the optical density of the β-tubulin band in the same lane. Similar levels of β-tubulin protein were expressed in dorsomedial and ventral thalamus samples both from patients with schizophrenia and control subjects (Fig. 1c). There was no main effect for diagnosis ($F = 0.22$; d.f. = 1,46; $p = 0.64$) and region ($F = 0.34$; d.f. = 1,46; $p = 0.56$), and no diagnosis × region interaction ($F = 2.60$; d.f. = 1,46; $p = 0.11$). There were no correlations between β-tubulin levels and age, tissue pH or post-mortem interval.

NMDA receptor subunit protein expression in dorsomedial and ventral thalamus

Antibodies directed against the NMDA receptor subunits NR1, NR2A and NR2B all detected single prominent bands at the appropriate molecular weights (180 kDa for NR2B, 165 kDa for NR2A and 120 kDa for NR1; Fig. 2a). There was no main effect of diagnosis ($F = 1.82$; d.f. = 1,46; $p = 0.18$) or region ($F = 1.37$; d.f. = 1,46; $p = 0.25$) on NR2B protein levels, but there was a significant diagnosis × thalamic sub-region interaction ($F = 6.71$; d.f. = 1,46; $p < 0.05$) (Fig. 2b). Post-hoc analysis revealed significantly increased levels of NR2B protein in the subjects with schizophrenia relative to comparison subjects in the dorsomedial thalamus ($p < 0.05$; Fisher's PLSD), while NR2B expression in ventral thalamus was unchanged (Fig. 2b). NR1 and NR2A protein expression was not different between groups in either dorsomedial or ventral thalamus (Fig. 2b). There was no main effect of diagnosis ($F = 1.52$; d.f. = 1,46; $p = 0.22$) or region ($F = 0.48$; d.f. = 1,46; $p = 0.49$) on NR1 protein levels and similarly, no main effect of diagnosis ($F = 1.40$; d.f. = 1,46; $p = 0.24$) or region ($F = 0.45$; d.f. = 1,46; $p = 0.50$) on NR2A protein levels. There was no diagnosis × region interaction for NR1 ($F = 2.93$; d.f. = 1,46; $p = 0.09$) or NR2A ($F = 1.20$; d.f. = 1,46; $p = 0.28$). None of the NMDA receptor subunit protein levels correlated with age, tissue pH or post-mortem interval.

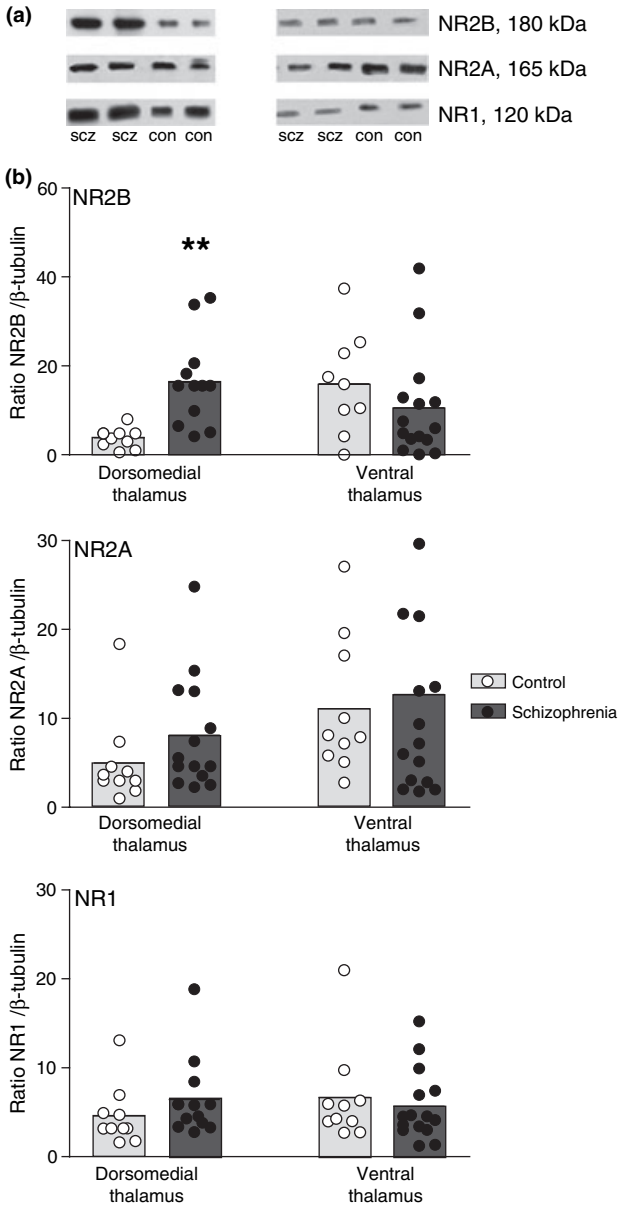

Fig. 2 NMDA receptor subunit expression in the dorsomedial and ventral thalamus in schizophrenia. (a) Representative immunoblots for NMDA receptor subunits NR2B, NR2A and NR1 using dorsomedial and ventral thalamus samples from a patient with schizophrenia (scz) and a control subject (con). Lanes show side-by-side duplicates of each subject. (b) There was no main effect of diagnosis for NR2B protein levels (upper panel), but there was a significant diagnosis × thalamic sub–region interaction ($F = 6.71$; d.f. = 1,46; $p < 0.05$). Post-hoc analysis revealed significantly increased levels of NR2B protein in the dorsomedial thalamus of subjects with schizophrenia compared with control subjects (**$p < 0.05$), but were unchanged in the ventral thalamus. NR2A (middle panel) and NR1 (lower panel) protein expression was not significantly different between diagnostic groups in either thalamic sub-region. Data are expressed as a ratio of the optical density value for the protein of interest over the optical density band for β-tubulin from the same patient.

NMDA receptor-associated post-synaptic protein expression in the thalamus in schizophrenia

Antibodies directed against post-synaptic density proteins SAP102, PSD95 and NF-L all detected single prominent bands at the appropriate molecular weights (102 kDa for SAP102, 95 kDa for PSD95 and 68 kDa for NF-L; Fig. 3a). There was no main effect of diagnosis ($F = 0.91$; d.f. = 1,46; $p = 0.35$) or region ($F = 0.34$; d.f. = 1,46; $p = 0.56$) for PSD95 protein levels, but there was a significant diagnosis × thalamic sub-region interaction ($F = 5.61$; d.f. = 1,46; $p < 0.05$; Fig. 3b). Post-hoc analysis revealed that PSD95 protein expression was increased in the dorsomedial thalamus of patients with schizophrenia compared with control subjects ($p < 0.05$; Fisher's PLSD), but was unchanged in the ventral thalamus (Fig. 3b). NF-L and SAP102 protein levels were not different between diagnostic groups. There was no main effect of diagnosis ($F = 1.09$; d.f. = 1,46; $p = 0.30$) or region ($F = 0.44$; d.f. = 1,46; $p = 0.51$) on NF-L protein levels. There was no main effect of diagnosis ($F = 1.36$; d.f. = 1,46; $p = 0.25$) on SAP102 protein levels, but there was a main effect of thalamic sub-region on SAP102 levels ($F = 7.31$; d.f. = 1,46; $p < 0.05$). Post-hoc analysis revealed that SAP102 protein expression was greater in ventral thalamus compared with dorsomedial thalamus (Fig. 3b). There was no diagnosis × region interaction for NF-L ($F = 2.32$; d.f. = 1,46; $p = 0.13$) or SAP102 ($F = 0.41$; d.f. = 1,46; $p = 0.52$). PSD95, NF-L and SAP102 protein levels did not correlate with age, tissue pH or post-mortem interval.

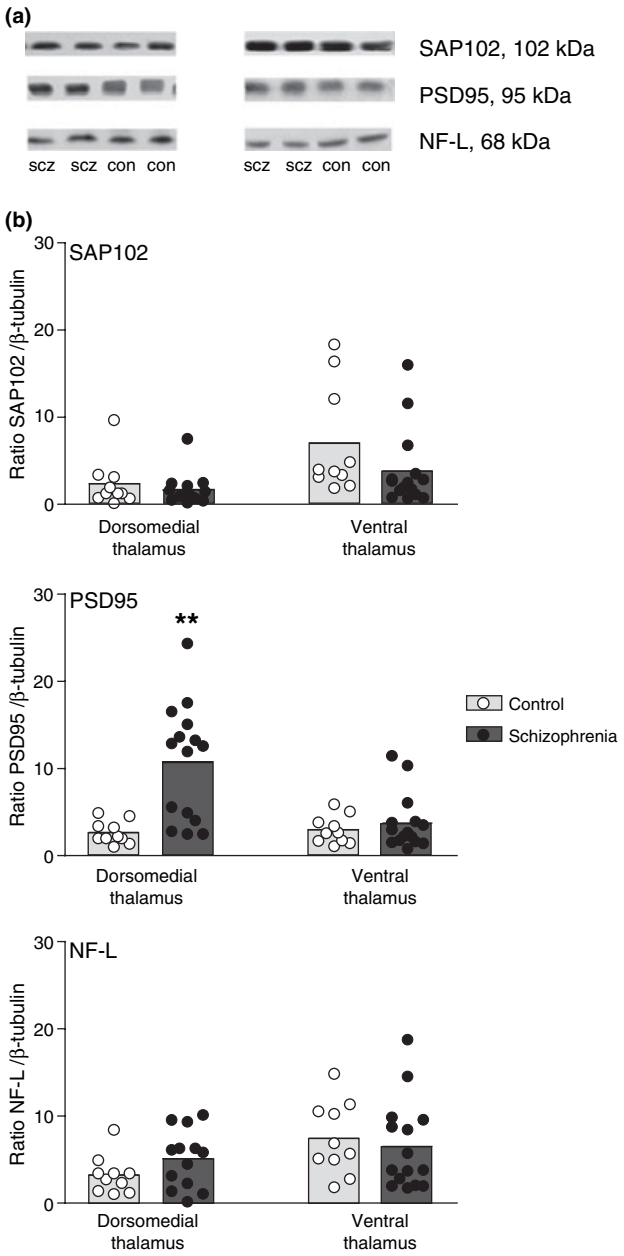

Fig. 3 Post-synaptic density protein expression in the dorsomedial and ventral thalamus in schizophrenia. (a) Representative immunoblots for NMDA receptor-associated post-synaptic proteins SAP102, PSD95 and NF-L using dorsomedial and ventral thalamus samples from a patient with schizophrenia (scz) and a control subject (con). Lanes show side-by-side duplicates of each subject. (b) There was no main effect of diagnosis for PSD95 protein levels (middle panel), but there was a significant diagnosis × thalamic sub–region interaction ($F = 5.61$; d.f. = 1,46; $p < 0.05$). Post-hoc analysis revealed that PSD95 protein expression was increased in the dorsomedial thalamus of patients with schizophrenia compared with control subjects (*$p < 0.05$), but was unchanged in the ventral thalamus. NF-L (lower panel) and SAP102 (upper panel) protein levels were not different between diagnostic groups. There was a main effect of thalamic sub-region for SAP102 levels ($F = 7.31$; d.f. = 1,46; $p < 0.05$), with post-hoc analysis revealing that SAP102 protein expression was greater in ventral thalamus compared with dorsomedial thalamus. Data are expressed as a ratio of the optical density value for the protein of interest over the optical density band for β-tubulin from the same patient.

Discussion

While there are numerous reports of structural and functional abnormalities of the thalamus in schizophrenia, surprisingly few studies have examined potential neurochemical abnormalities that may accompany these pathological changes (Clinton and Meador-Woodruff 2004a). Our recent work has revealed gross disturbances of multiple molecules involved in glutamate neurotransmission, including changes in NMDA receptor subunit transcripts and binding sites (Ibrahim *et al*. 2000), receptor-associated PSD protein transcripts (Clinton *et al*. 2003), and transcripts encoding pre-synaptic and glial-associated glutamate transporters (Smith *et al*. 2001a,b) in the thalamus of elderly patients with schizophrenia. In the present study, we performed western blot analysis and observed increased protein expression of the

NR2B NMDA receptor subunit and its associated intracellular protein, PSD95, in the dorsomedial thalamus of patients with schizophrenia compared with control subjects. While the pattern of protein expression changes does not entirely correspond to our previously reported transcript and binding data, these findings provide additional evidence of altered glutamate neurotransmission in the thalamus in schizophrenia, and may reflect a complicated array of intracellular disturbances that contribute to thalamic dysfunction in schizophrenia.

NMDA receptor abnormalities in the thalamus in schizophrenia

Our previous work showed reduced expression of NR1 and NR2C NMDA receptor subunit transcripts in the thalamus in schizophrenia (Ibrahim *et al*. 2000). Receptor autoradiography studies revealed reduced expression of the glycine binding site (located on the NR1 subunit) and polyamine binding site (located on the NR2B subunit), although other binding sites, including the intrachannel phencyclidine (PCP) binding site, were unchanged (Ibrahim *et al*. 2000). These data suggest that while the total number of NMDA receptors expressed at the cell surface is unchanged in schizophrenia, there may be a shift in subunit stoichiometry such that there are fewer NR1 and NR2B subunits incorporated into thalamic NMDA receptor channels (Ibrahim *et al*. 2000). Our present western blot results show increased NR2B NMDA receptor subunit protein in the dorsomedial thalamus of patients with schizophrenia relative to comparison subjects (Fig. 2b). One possible explanation for the reduction of receptor binding together with apparently increased amounts of NR2B protein could be that a mutation in either the NR1 or NR2B genes in schizophrenia produces abnormal proteins. Several studies have investigated possible genetic mutations of NMDA receptor subunits but, by and large, these studies have been unable to find strong associations between NMDA receptor subunit alterations and schizophrenia (Harrison and Weinberger 2005). Thus, this does not readily explain our discordant protein and binding data.

An alternative explanation for the apparent discrepancy between binding and protein data is that increased NR2B protein is produced in the dorsomedial thalamus but is not successfully incorporated into receptors, which are delivered and inserted into the synaptic membrane. There is a multitude of intracellular mechanisms that appear to regulate the assembly of NMDA receptor subunits into functional channels, endoplasmic reticulum retention or release of those receptors, receptor trafficking within the cell and eventual synaptic delivery (McIlhinney *et al*. 1998; Setou *et al*. 2000; Standley *et al*. 2000; Steigerwald *et al*. 2000; Scott *et al*. 2001; Barria and Malinow 2002; Carroll and Zukin 2002; Fukaya *et al*. 2003; Guillaud *et al*. 2003; Sans *et al*. 2003; Washbourne *et al*. 2004). The present western data suggest that while there are high levels of NR2B protein available, there may be problems with NMDA receptor assembly, endoplasmic reticulum release and/or trafficking that prevent NR2B-containing receptors from successfully reaching the synaptic membrane. Animal work using a selective knockout of the NR1 gene provides *in vivo* evidence demonstrating that disrupting NMDA receptor subunit composition impairs normal subcellular targeting of NMDA channels (Fukaya *et al*. 2003). Fukaya and co-workers demonstrated that NR1 gene deletion in the CA1 region of the hippocampus leads to a reduction of NR2B protein expressed in the dendrites, and an accumulation of NR2B subunit protein in the perikarya where it is condensed in the lumen of the endoplasmic reticulum (Fukaya *et al*. 2003). Based on our present findings, we hope to pursue studies which will examine the subcellular compartmentalization of NMDA receptor subunits in schizophrenia in order to determine whether certain subunits (e.g. NR2B subunits) are abnormally concentrated in the endoplasmic reticulum, rather than incorporated in receptors expressed at the synaptic membrane. This line of investigation would offer an intriguing new perspective on dysregulated NMDA receptor functioning in schizophrenia.

Altered PSD95 protein expression in the thalamus in schizophrenia

Previous *in situ* hybridization studies revealed increased expression of PSD95, NF-L and SAP102 transcripts in the thalamus in schizophrenia (Clinton *et al*. 2003). Results from our current western blot studies show increased PSD95 protein expression in the dorsomedial thalamus in schizophrenia, but no changes in NF-L and SAP102 protein levels (Fig. 3b). PSD95 and other NMDA receptor-associated PSD proteins generally promote NMDA receptor function in a variety of ways, including participating in subcellular targeting and trafficking of receptors to the post-synaptic membrane (Standley *et al*. 2000; Scott *et al*. 2001; Sans *et al*. 2003), modulating NMDA receptor sensitivity to glutamate (Rutter and Stephenson 2000) and linking the receptor to various downstream intracellular signaling pathways (Sheng and Pak 2000; Sheng 2001) (Fig. 4). Our transcript, binding and western data all point to dysregulated NMDA receptor function in the thalamus in schizophrenia. As PSD95 generally acts to promote NMDA receptor function, and NMDA receptor activity has been shown to modulate PSD95 expression (Linden *et al*. 2001; Wyneken *et al*. 2001), thalamic NMDA receptor dysfunction in schizophrenia could conceivably stimulate a compensatory up-regulation of PSD95 transcript and protein expression.

Although our previous transcript data revealed increased NF-L and SAP102 mRNA levels in the thalamus of patients with schizophrenia (Clinton *et al*. 2003), our present western blot studies showed no difference in either NF-L or SAP102 protein expression in dorsomedial or ventral thalamus (Fig. 3b). NF-L has been shown to interact with the NR1 NMDA receptor subunit (Ehlers *et al*. 1998) and SAP102

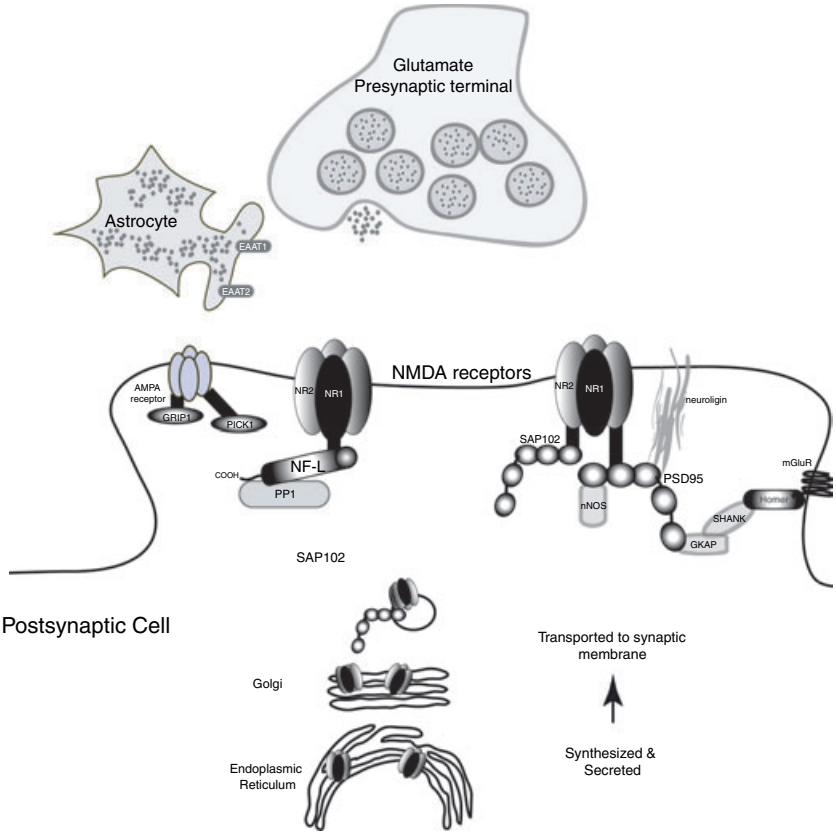

Fig. 4 Schematic diagram of a NMDA receptor subunits interacting with post-synaptic density proteins. NMDA receptor subunits have long C-terminal intracellular tails that bind to post-synaptic proteins like PSD95, SAP102 and NF-L, which promote NMDA receptor function, in part, by linking receptors to the cytoskeleton and to specific signal transduction molecules such as neuronal nitric oxide synthase (nNOS), protein phosphatase 1 (PP1), and guanylate kinase-associated protein (GKAP). PSD proteins also appear to participate in sub-cellular trafficking receptors and help to stabilize them in the synaptic membrane after they are inserted. AMPA and metabotropic glutamate receptors (mGluRs) interact with similar families of molecules, including the AMPA receptor-associated PICK1 and GRIP1 proteins, and Homer, which interacts with mGluRs.

interacts with the NR2 subunits (Lau *et al*. 1996; Muller *et al*. 1996; Sans *et al*. 2003). Thus, it is possible that NMDA receptor dysfunction in the thalamus stimulates a compensatory increase in transcript expression, as it does for PSD95. Unlike PSD95, however, the increased amounts of NF-L and SAP102 transcripts do not appear to be translated into increased amounts of protein.

One possible explanation for the failure to find parallel changes in protein and mRNA is that there might be a dissociation between the cellular localization of transcript and protein. Transcripts are exclusively expressed in the cell body, while protein is expressed in the soma, axons and dendrites. Approximately 30–40% of cells in the dorsal thalamus are inhibitory interneurons which reside wholly in the thalamus, and the remaining 60–70% of thalamic cells consists of large glutamatergic relay neurons which send long projections to, and receive reciprocal input from, cortical and subcortical structures (Steriade *et al*. 1997). NMDA receptor subunit transcripts are predominantly expressed by relay neurons (Jones 1998), and our cell-level transcript studies suggest that PSD protein transcripts are also concentrated in relay neurons (Clinton *et al*. 2003). While a large percentage of translated NF-L and SAP102 protein may remain in the cell body and/or dendrites of thalamic relay cells, some of it may be transported distally to axonal sites outside the thalamus and thus, it would not be contained in our dissection of the dorsomedial thalamus. Furthermore, our dissected tissue would not only include relay cell soma and dendrites and entire inhibitory interneurons, but also afferent fibers and terminals coming into the thalamus from elsewhere in the brain. If cells projecting into the thalamus produced and expressed abnormal amounts of PSD protein in these distal fibers, it could contribute to this discrepancy between observed mRNA and protein levels.

Another possibility to consider is that even with normal (e.g. NF-L and SAP102) or elevated (e.g. PSD95) amounts of total protein available, these post-synaptic proteins may not properly interact with NMDA receptors, which would further contribute to NMDA receptor dysfunction in schizophrenia. Co-immunoprecipitation studies might be useful to pursue the idea that abnormalities of the NMDA receptor signaling complex in schizophrenia may involve a perturbation of protein–protein interactions between NMDA receptor subunits and their associated post-synaptic density proteins.

Limbic regions of thalamus appear to be most affected in schizophrenia

Our present western blot results show increased protein expression for the NR2B NMDA receptor subunit and its associated post-synaptic density protein, PSD95, in the dorsomedial, but not ventral, region of the thalamus. Figure 1(a) outlines the two thalamic subregions dissected

for each patient and comparison subject. The dorsomedial dissected region contained portions of the dorsomedial nucleus, anterior nucleus and central medial nucleus of the thalamus, while the ventral region contained portions of the ventral anterior and ventral lateral nuclei of the thalamus. Both magnetic resonance imaging studies and post-mortem morphometric studies have reported nuclei-specific abnormalities in schizophrenia, including decreased volume and cell number in the dorsomedial nucleus (Pakkenberg 1990, 1992, 1993; Popken *et al*. 2000; Young *et al*. 2000; Byne *et al*. 2001; Gilbert *et al*. 2001; Byne *et al*. 2002; Danos *et al*. 2003), anterior thalamic nucleus (Danos *et al*. 1998; Young *et al*. 2000) and central medial nucleus (Gilbert *et al*. 2001). Furthermore, our earlier *in situ* hybridization and receptor autoradiography studies showed the most prominent NMDA receptor abnormalities to be in the dorsomedial and central medial nuclei, both of which reciprocally project to the prefrontal cortex and other limbic structures (Ibrahim *et al*. 2000). Therefore, it is perhaps not surprising that our most robust protein changes also occur in these limbic-associated nuclei, rather than the non-limbic-associated ventral tier nuclei.

Limitations of this study

Several limitations need to be considered when interpreting data from these studies. First, an important limitation of this and all post-mortem studies in schizophrenia is the possible confounding effect of anti-psychotic medications, as these drugs potentially regulate the neurochemical systems under study. All the patients with schizophrenia used in this study were exposed to anti-psychotic medication for decades, and it is therefore possible that our results are related to anti-psychotic exposure rather than being associated with the disease process itself. While studies in both human and rat brain have shown that anti-psychotic medications modulate thalamic activity (Deutch *et al*. 1995; Cohen and Wan 1996; Holcomb *et al*. 1996; Cohen and Yurgelun-Todd 2001), rodent studies suggest that typical anti-psychotics do not affect the expression of transcripts encoding NMDA receptor subunits and associated PSD molecules (unpublished observations), or thalamic NMDA receptor protein expression (Ulas *et al*. 1993). The present study is also limited by the small sample size used. Finally, these data are from elderly subjects so, while the resulting data represent thalamic neurochemical changes that exist in the late stages of schizophrenia, they may not generalize to younger patients. In fact, other work in our laboratory has shown that, at least at the level of transcript expression, middle-aged and elderly patients with schizophrenia show divergent patterns of glutamatergic molecular abnormalities in the thalamus, suggesting that different stages of the disease are associated with different neurochemical changes (Clinton and Meador-Woodruff 2004b). Therefore, although these findings are intriguing, it is as yet unclear whether they are primarily due to schizophrenia or are a by-product of having this chronic illness for many decades.

Conclusions

Several lines of evidence support a hypothesis of glutamatergic dysfunction in schizophrenia, including our reports of altered NMDA receptor subunit and PSD protein transcript expression in the thalamus (Ibrahim *et al*. 2000; Clinton *et al*. 2003). The present western blot studies reveal increased expression of the NR2B NMDA receptor subunit and its associated post-synaptic density protein, PSD95, in the dorsomedial thalamus of patients with schizophrenia, providing further evidence of NMDA receptor abnormalities in schizophrenia. More importantly, however, these data suggest that glutamatergic abnormalities in the illness may involve myriad intracellular changes, which may include altered sub-cellular trafficking of the NMDA receptor, or a breakdown of glutamate receptor signaling pathways due to insufficient linkage between NMDA receptors and their associated PSD proteins. PSD95-like molecules, which exist for several other neurotransmitter receptor families besides glutamate receptors (see Lezcano *et al*. 2000; Lidow *et al*. 2001; Koh *et al*. 2003; Clinton *et al*. 2005), provide a substrate for cross-talk between different neurotransmitter systems (e.g. glutamate and dopamine); thus, abnormalities of PSD95 and other key intracellular molecules may be crucial to understanding molecular neurobiological mechanisms involved in schizophrenia.

Acknowledgements

This work was supported by MH70895 (JHM-W), and MH64673, MH45212 and VA-Merit (VH), and was presented at the Society of Neuroscience 33rd Annual Meeting, New Orleans, LA, in 2003.

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