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Chronic Exposure of Rats to Cognition Enhancing Drugs Produces a Neuroplastic Response Identical to that Obtained by Complex Environment Rearing

Keith J Murphy*[,1], Andrew G Foley[1], Alan W O'Connell[1] and Ciaran M Regan[1]

[1]*Department of Pharmacology, Applied Neurotherapeutics Research Group, Conway Institute, University College Dublin, Belfield, Dublin, Ireland*

Recent data suggest that Alzheimer's patients who discontinue treatment with cholinesterase inhibitors have a significantly delayed cognitive decline as compared to patients receiving placebo. Such observations suggest cholinesterase inhibitors to provide a disease-modifying effect as well as symptomatic relief and, moreover, that this benefit remains after drug withdrawal. Consistent with this suggestion, we now demonstrate that chronic administration of tacrine, nefiracetam, and deprenyl, drugs that augment cholinergic function, increases the basal frequency of dentate polysialylated neurons in a manner similar to the enhanced neuroplasticity achieved through complex environment rearing. While both drug-treated and complex environment reared animals continue to exhibit memory-associated activation of hippocampal polysialylated neurons, the magnitude is significantly reduced suggesting that such interventions induce a more robust memory pathway that can acquire and consolidate new information more efficiently. This hypothesis is supported by our findings of improved learning behavior and enhanced resistance to cholinergic deficits seen following either intervention. Furthermore, the level of enhancement of basal neuroplastic status achieved by either drug or environmental intervention correlates directly with improved spatial learning ability. As a combination of both interventions failed to further increase basal polysialylated cell frequency, complex environment rearing and chronic drug regimens most likely enhanced cognitive performance by the same mechanism(s). These findings suggest that improved memory-associated synaptic plasticity may be the fundamental mechanism underlying the disease modifying action of drugs such as cholinesterase inhibitors. Moreover, the molecular and cellular events underpinning neuroplastic responses are identified as novel targets in the search for interventive drug strategies for the treatment of neurodegenerative and neuropsychiatric disorders.
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INTRODUCTION

Many different approaches have suggested promise in the treatment of Alzheimer's disease, but only therapies designed to boost central cholinergic transmission have advanced to clinical use. Clinical trials examining cholinergic augmentation with acetylcholinesterase inhibitors (AChEIs), such as tacrine, rivastigmine, donepezil, and galantamine, have consistently detected symptomatic improvement of cognitive impairment in Alzheimer's disease (Irizarry and Hyman, 2001). Symptomatic improvement in these studies was modest. Relative to placebo, following 6 months of treatment, patients improved by 2.8–6.0 points on the 70 point Cognitive Subscale of the Alzheimer's Disease Assessment Scale (ADAS-Cog; Knapp *et al*, 1994; Rogers *et al*, 1998; Rosler *et al*, 1999; Raskind *et al*, 2000; Burns *et al*, 2004).

Due to the unfavorable side effect profile of AChEIs, other drug classes, with distinctly different primary mechanisms of action, have been investigated and shown promise in the treatment of cognitive deficits associated with Alzheimer's disease. Moreover, several such potential Alzheimer's treatments have been shown to augment cholinergic function. For example, the nootropic antidementia agent nefiracetam stimulates nicotinic receptors as efficiently as galantamine and donepezil, an effect that is dependent on Gs protein(s) (Nishizaki *et al*, 2000; Shorvon, 2001; Narahashi *et al*, 2004). Also, the procognitive action of the monoamine oxidase B inhibitor, l-deprenyl, in aged rats and Alzheimer's patients is thought to result from monoaminergic-driven enhancement of cholinergic function, in particular, in the hippocampus (Tariot *et al*, 1987; Piccinin *et al*, 1990; Mangoni *et al*, 1991; Molinengo and Ghi, 1997).

*Correspondence: Dr KJ Murphy, Department of Pharmacology, Applied Neurotherapeutics Research Group, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland, Tel: +353 1 7166778, Fax: +353 1 7166920, E-mail: keith.murphy@ucd.ie
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Thus, interventions with distinct primary targets may ultimately influence a common cholinergic pathway to mediate symptomatic relief.

Recent data suggest that patients who discontinue treatment with cholinesterase inhibitors have a significantly delayed cognitive decline as compared to patients receiving placebo (Farlow *et al*, 2000). Such observations suggest AChEIs to provide a disease-modifying effect as well as symptomatic relief and, moreover, that this benefit remains after drug withdrawal. Furthermore, it remains unclear if such drug-derived benefit is mechanistically similar to the protective action of environmental factors such as higher educational attainment known to correlate with reduced risk of Alzheimer- and Parkinson-related dementia (Snowdon *et al*, 1996; Glatt *et al*, 1996). It is not unreasonable to assume that both environmental and AChEI disease-modifying action(s) require persisting alteration in molecular mechanisms that underpin cognitive function in the medial temporal lobe, as these structures are particularly sensitive to degeneration in Alzheimer's disease (Terry *et al*, 1991; Dickson *et al*, 1995; Gomez-Isla *et al*, 1996; Krishnan *et al*, 2003). Associating molecular mechanisms with this drug-derived benefit provides an important step in the development of more effective therapeutic strategies for neurodegenerative conditions such as Alzheimer's disease.

Significant evidence exists to suggest that memory consolidation requires synaptic reorganization and, in particular, modulation of cell adhesion within the hippocampus (for reviews see Regan, 2004; Lamprecht and LeDoux, 2004). For example, the neural cell adhesion molecule (NCAM) is necessary for activity-dependent synaptic plasticity, such as that associated with hippocampal long-term potentiation (LTP) and avoidance conditioning and spatial learning paradigms (Doyle *et al*, 1992; Lüthi *et al*, 1994; Rønn *et al*, 1995; Alexinsky *et al*, 1997). Glycosylation of NCAM with extended chains of α2,8-linked polysialic acid (PSA) is also necessary for memory consolidation as their cleavage with endoneuraminidase prevents LTP and spatial learning (Becker *et al*, 1996; Muller *et al*, 1996; Kleene and Schachner, 2004). Within the hippocampus, a transient increase in polysialylation of neurons located at the dentate infragranular zone at 10–12 h following learning is necessary for memory consolidation (Fox *et al*, 1995; Murphy *et al*, 1996; Foley *et al*, 2003; Sandi *et al*, 2003). This neuroplastic mechanism is required for dendritic remodelling (Nothias *et al*, 1997; Hoyk *et al*, 2001) and is likely to be an important factor in the elaboration and integration of circuitry associated with the consolidation of novel behavioral repertoires (Wang *et al*, 2000).

Consistent with the existence of a common mechanism underlying an inherent disease-modifying action from augmentation of cholinergic function, we now demonstrate chronic administration of tacrine, nefiracetam, and deprenyl to increase the basal frequency of dentate polysialylated neurons in a manner similar to the enhanced neuroplasticity achieved through complex environment rearing. Moreover, reduced requirement for neuroplastic activation, improved maze learning, and increased resilience against cholinergic deficits accompany the enhanced NCAM PSA expression in the hippocampus following drug or environmental intervention.

MATERIALS AND METHODS

Source of Animals

Male Wistar rats were purpose bred and housed in the Biomedical Facility, University College Dublin. These were maintained in a 12 h light/dark cycle with *ad libitum* access to food and water.

Effects of Chronic Drug Treatment or Complex Environment Rearing on Basal NCAM PSA Expression in the Hippocampal Dentate Gyrus

Chronic drug treatment. For studies of drug action on basal NCAM PSA expression, postnatal day 40 animals were housed singly and assigned randomly to groups receiving nefiracetam, tacrine, deprenyl, phenytoin, NNC-711 (Figure 1), or 0.9% (w/v) saline vehicle in which all drugs were prepared. The doses employed for each drug and the animal numbers for this first phase are detailed in Table 1. Where appropriate, these doses were chosen to reflect the clinical equivalent (McNamara, 2001; Standaert and Young, 2001). The animals were dosed daily via intraperitoneal injection from postnatal day 41 to postnatal day 80. Just prior to drug administration, rat weight gain was monitored for possible adverse effects of treatment. Only the highest dose of tacrine exhibited a blunting of the growth curve, as might be expected from the hepatotoxicity associated with tacrine at this and higher doses (Blackard *et al*, 1998). However, no drug-induced abnormalities of open-field behavior were observed on any of the 3 days prior to the day of training (data not shown).

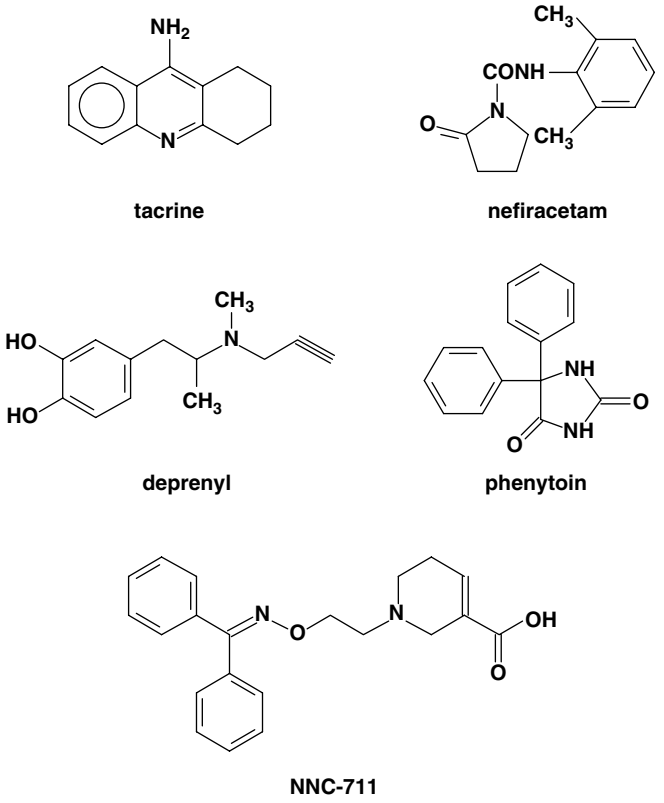

Figure 1 Chemical structures of drugs used in study.

Table 1 Chronic Drug Treatments Employed in Phase 1

Drug class	Treatment	Dose	*n*
Control	Saline	0.9% (w/v)	6
Nöotropic	Nefiracetam	3.0 mg/kg	6
		9.0 mg/kg	6
		15.0 mg/kg	5
		30.0 mg/kg	5
Cholinomimetic	Tacrine	1.0 mg/kg	7
		3.0 mg/kg	7
		6.0 mg/kg	4
MAO-B inhibitor	Deprenyl	5.0 mg/kg	7
		10.0 mg/kg	7
Na^+ channel blocker	Phenytoin	4.0 mg/kg	7
		8.0 mg/kg	7
GABAmimetic	NNC-711	1 mg/kg	6

The *n* number refers to initial studies characterizing the basal NCAM PSA expression only. Further animals were treated for studies on learning behavior, learning induced activation of NCAM polysialylation, and complex environment combination studies.

Complex environment rearing. Male Wistar rats were raised from weaning (postnatal day 25) to postnatal day 80 in complex environment or social groups. The complex environment consisted of a large purpose built ($1\,m^3$) stainless-steel cage in two sections connected by a ramp. The cage was filled with items designed to generate environmental stimulation, for example: wooden/plastic blocks, wood chips, plastic containers, tunnels, exercise wheels, balls, tubes, see-saws, water-pools, wheeled vehicles, alternative housing, hanging chimes, tight-ropes and bridges, rolling chimes, and mirrors. The composition and placement of these items was altered daily. In addition, extra walls and floors were inserted into the cage and the location of food and water sources changed daily. Animals were raised in this environment in groups of 30. Social control animals were raised in groups of 15 under standard conditions that consisted of a runner cage ($100 \times 50 \times 30\,cm^3$) containing no enrichment but with *ad libitum* access to food and water.

Immunohistochemistry. Upon killing, the whole brain was quickly dissected out, coated immediately in an optimal cutting temperature compound (Gurr, UK), snap-frozen in liquid nitrogen-cooled n-hexane, and stored at $-80^{\circ}C$ until required for further processing. Horizontal sections of 12 μm were cut from frozen brain tissue using a MICROM (Series 500) cryostat. Serial sections were obtained for analysis from a point −5.6 mm from Bregma (Paxinos and Watson, 1986) and thaw-mounted onto 0.1% (w/v) poly-l-lysine coated glass slides. The brain sections were fixed in 70% (v/v) ethanol for 30 min, washed twice for 10 min in a washing buffer of 0.1 M phosphate-buffered 0.9% saline, pH 7.4 (PBS), and incubated overnight (20 h) in a humidified chamber at room temperature with anti-PSA (generous gift of Professor G Rougon; Rougon *et al*, 1986). This primary antibody was diluted 1 : 500 in an incubation buffer composed of PBS containing 1% w/v bovine serum albumen (Sigma Chemical Co., UK) and 1% v/v normal goat serum (DAKO, Denmark) in order to eliminate nonspecific staining. The sections were washed again and exposed for 3 h to fluorescein-conjugated goat anti-mouse IgM (Calbiochem, UK) diluted 1 : 100 with incubation buffer. The sections received a final wash before being mounted in Citifluor® (Agar, UK), a fluorescence-enhancing medium.

The staining pattern was observed with a Leitz DM RB fluorescence microscope using an exciting wavelength of 495 nm and an emitting wavelength of 525 nm. Immunofluorescence staining was specific as it was eliminated completely by omission of either the primary or secondary antibody and by preabsorbing anti-PSA with colominic acid (1 mg/ml; Sigma Chemical Co., UK), which contains α2,8-linked homopolymers of sialic acid (Murphy *et al*, 1996). Where relevant, sections were counterstained by a brief exposure (60 s) to propidium iodide (50 ng/ml PBS) that was detected using an excitation wavelength of 552 nm and an emission wavelength of 570 nm. The total number of PSA-immunoreactive neurons in the dentate granule cell layer and at the hilar border were counted in seven alternate 12 μm sections commencing 5.6 mm below Bregma, to preclude double counting of the 5–10 μm perikarya. Cell counts were divided by the total area of the granule cell layer, which included all propidium iodide labelled cells, and multiplied by the average granule cell layer area, which was $0.15 \pm 0.01\,mm^2$ at this level, and the mean calculated for each animal. These means were used to establish the mean ± SEM for each animal group. Area measurements of propidium iodide stained granule cell perikarya were performed using a Quantimet 500 Image Analysis System. Parametric statistical comparisons were made using the Student's *t*-test and *p*-values <0.05 were taken to be significant.

Effect of Chronic Drug Treatment or Complex Environment Rearing on Learning Ability and Memory-Associated Activation of NCAM PSA Expression

For these learning experiments, a second cohort of animals were chronically exposed to tacrine, nefiracetam, and deprenyl, at doses of 1, 15, and 5 mg/kg, respectively, or saline (0.9% w/v) from postnatal day 41 to 79. These doses were selected as they produced the greatest increase in basal NCAM PSA expression in phase 1. These animals were then divided into untrained naives ($n=3$) and groups to be trained in either water maze ($n=5$) or passive avoidance ($n=5$) learning paradigms. Thus, as the animals were drug-free on day of training (postnatal day 80), no effects observed were attributable to acute drug action. In addition, social and complex environment groups raised together with the untrained animals analyzed in phase 1 were trained in either water maze ($n=5$) or passive avoidance ($n=5$).

Passive avoidance training. On postnatal day 80, animals were trained in a one-trial, step-through, light–dark passive avoidance paradigm (Fox *et al*, 1995). The smaller, illuminated compartment was separated from a larger compartment by a shutter that contained a small entrance. The floor of the training apparatus consisted of a grid of stainless-steel bars that could deliver a remotely controlled, scrambled shock (0.75 mA every 0.5 ms) for 5 s when the animal entered the dark chamber. At 12 h post-training, the animals were tested for recall of this inhibitory stimulus by placing them into the light compartment and noting their latency to enter the dark compartment. A criterion period of 600 s was used. Nonparametric statistical comparisons were made using the Mann–Whitney *U*-test and *p*-values <0.05 were considered to be significant. Animals were killed immediately following the 12 h recall test and their brains prepared and processed as above for immunohistochemical analysis of NCAM PSA expression.

Water maze training. On postnatal day 80, animals were trained in the water maze. This spatial learning task has been described in detail previously (Murphy *et al*, 1996). Briefly, the water maze apparatus consisted of a large circular pool (1 m diameter, 80 cm high, temperature $26\pm1^{\circ}$C) with a platform (11 cm diameter) submerged 1.5 cm below the water surface. Both the pool and the platform were constructed of black polyvinyl plastic and offered no intra-maze cues to guide escape behavior. The experimental room contained several extra-maze visual cues. During training the platform was hidden in the same quadrant 30 cm from the edge of the maze. Each of the five trials started with the rat facing the wall of the maze at one of three locations. The time taken by the rat to find the hidden platform within a 60 s period was recorded. On the first trial, rats failing to find the platform within the 60 s period were placed on it for 10 s. Times to the platform were measured over five trials in the training session with an inter-trial interval of 300 s. All data were calculated and graphed as mean $\pm$ SEM and the presence of significant difference between conditions was determined by two-way ANOVA and *post hoc* Bonferroni analysis (GraphPad Prism 4 software). In all cases, values of $p<0.05$ were deemed to be significant. Animals were killed 12 h following the last trial and their brains prepared and processed as above for immunohistochemical analysis of NCAM PSA expression.

Correlation analysis. To seek a relationship between enhancement of basal hippocampal neuroplasticity and learning ability, these parameters were compared by Pearson correlation (GraphPad Prism 4 software; $p<0.05$ indicated significant correlation). Here, for each condition, the percent change in the basal NCAM PSA-positive cell number following the intervention (Table 2) was taken as an index of effect on neuroplasticity status while the mean latency to platform across all animals and all trials was taken as an index of spatial learning ability.

Effect of Chronic Drug Treatment or Complex Environment Rearing on Amnesic Action of Acetylcholine Antagonism

This study utilised the amnesic action of the acetylcholine antagonist scopolamine when administered 6 h postpassive avoidance training that we have previously described (Doyle and Regan, 1993). Briefly, animals were chronically exposed to nefiracetam (15 mg/kg/day) or saline (0.9% w/v) from postnatal day 41 to 79 or reared in social or complex environment as before. Again, as the animals were drug-free on day of training (postnatal day 80), no effects observed were attributable to acute drug action. On postnatal day 80, all animals were trained in passive avoidance as above and administered scopolamine (0.8–2.0 mg/kg; $3\leqslant n\leqslant 8$) 6 h after training. Each animal was tested for recall 24 h after training. An untreated control group was included to indicate normal avoidance recall latency. All data were calculated and graphed as mean $\pm$ SEM escape latency and the presence of significant difference between conditions was determined by two-way ANOVA and *post hoc* Bonferroni analysis (GraphPad Prism 4 software). In all cases, values of $p<0.05$ were deemed to be significant.

Table 2 Effect of Chronic Administration of Nefiracetam, Tacrine, and Deprenyl or Complex Environment Rearing on Basal Expression and Learning-Induced Activation of NCAM PSA

	Drug treatment				Rearing	
	Saline (%)	**Nefiracetam (%)**	**Tacrine (%)**	**Deprenyl (%)**	**Social (%)**	**Complex (%)**
Naïve						
% increase over control	0	28	18	16	0	33
Water maze						
% increase over basal	23	0	9	3	30	16
Passive avoidance						
% increase over basal	39	9	16	2	57	20

For naïve, the data are percent difference from control (saline-treated or social reared). For both training paradigms, data are percent difference from respective basal PSA-positive cell number ($3\leqslant n\leqslant 5$). The percent changes are worked out using the mean values from Figure 4. See Figure 4 for indications of significance and variance in the data.

Effect of Combining Chronic Drug Treatment and Complex Environment Rearing on NCAM PSA Expression

Animals were raised in either social or complex environment groups as before. Within each environment animal groups received either nefiracetam (15 mg/kg/day) or saline (0.9% w/v) from postnatal day 25 to 79 ($n = 4$ per treatment per environment). Animals were killed on postnatal day 80 and their brains prepared and processed as above for immunohistochemical analysis of NCAM PSA expression.

RESULTS

Chronic Drug Treatment or Complex Environment Rearing Increase Basal NCAM PSA Expression in the Hippocampal Dentate Gyrus

Chronic exposure to tacrine resulted in a significant increase in the frequency of polysialylated cells in the hippocampal dentate infragranular zone. This increase was observed with every dose employed, however, with the higher doses the polysialylated cell frequency tended to return to that observed in the saline control (Figure 2). This latter observation suggested a bell-shaped dose–response effect. Within the dentate gyrus, immunohistochemical procedures revealed the polysialylated cells to be located specifically at the infragranular zone with a well-established dendritic arbor extending through the granule cell zone and into the overlying molecular layer (Figure 3).

Nefiracetam, a well-established nöotrope (Shorvon, 2001) and the neuroprotective drug deprenyl (Ebadi *et al*, 2002) were employed to determine if increased dentate polysialylated cell frequency followed chronic administration of other agents that enhance acetylcholine and memory function. Nefiracetam exhibited an action very similar to that observed with tacrine. Increased polysialylated cell frequency was observed over a dose range of 9–30 mg/kg and the dose–response effect was bell-shaped (Figures 2 and 3). Furthermore, clinically relevant concentrations of deprenyl (5 and 10 mg/kg; Standaert and Young, 2001) when administered in a chronic manner resulted in a significant increase in dentate polysialylated cell frequency (Figures 2 and 3).

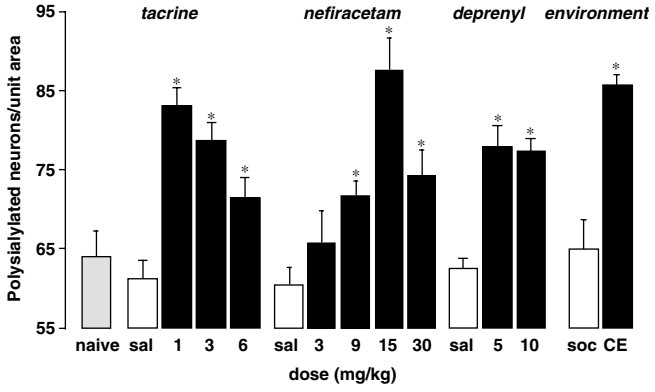

Figure 2 Influence of chronic treatment with nefiracetam, tacrine, deprenyl, or complex environment rearing on basal expression of polysialylated neurons in the hippocampal dentate infragranular zone. All data are the mean ± SEM number of cells/0.15 mm^2 of the granule cell layer ($4 \leqslant n \leqslant 7$). Values significantly different ($p < 0.05$; Student's *t*-test) from naïve are indicated with an asterisk. sal: saline-treated (0.9% w/v); soc: social housing; CE: complex environment.

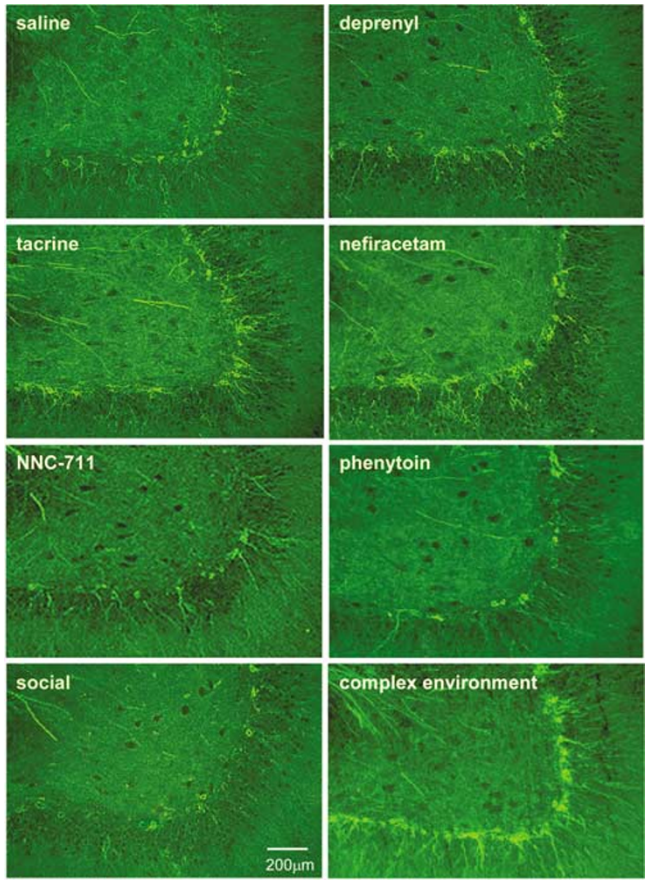

Figure 3 Photomicrographs illustrating the effect of chronic treatment with saline (0.9% w/v), deprenyl (5 mg/kg/day), tacrine (1 mg/kg/day), nefiracetam (15 mg/kg/day), NNC-711 (1 mg/kg/day), phenytoin (8 mg/kg/day) and rearing in a social or complex environment on the frequency of polysialylated hippocampal infragranular neurons in the postnatal day 80 male Wistar rat.

NNC-711, a GABA reuptake inhibitor and potent procognition agent that is not associated with enhanced cholinergic function, failed to increase basal polysialylated cell frequency (64.4 ± 3.9 *vs* 61.7 ± 7.8; not significant) at 1 mg/kg, the optimal dose of its bell-shaped cognition enhancing effect (O'Connell *et al*, 2001). Moreover, as may be expected, drugs without cognition-enhancing actions failed to increase polysialylated cell frequency following chronic administration. The anticonvulsant phenytoin was employed as it dampens neural activity through a sodium channel blocking mechanism and does not mediate cognition-enhancement (Rogawski and Porter, 1990; Kuo and Bean, 1994). At 4 mg/kg, a dose in the mid-therapeutic range, or at 8 mg/kg, a dosage approaching the upper limit of nontoxic levels (McNamara, 2001), chronic administration of phenytoin resulted in polysialylated cell counts of 64.3 ± 2.2 and 63.9 ± 2.4 cells/unit area, respectively, values not significantly different from those observed in vehicle-treated animals (62.8 ± 1.5 cells/unit area). Immunohistochemical procedures confirmed chronic administration of

NNC-711 and phenytoin to be without effect on the integrity of the polysialylated neurons. In all cases, their location and extent of dendritic arbor reaching to the molecular layer remained unchanged (Figure 3).

As increased basal dentate polysialylated cell frequency has previously been observed following complex environment rearing (Young *et al*, 1999), we employed this paradigm to compare its effect with that obtained by chronic drug administration. Maintaining animals in this environment from postnatal day 25 to postnatal day 80 resulted in a significant increase in the frequency of polysialylated neurons at the dentate infragranular zone (Figures 2 and 3). Significantly, the increase in polysialylated cell frequency was indistinguishable from that observed following chronic treatment with the optimal doses either of tacrine, deprenyl, or nefiracetam.

Chronic Drug Treatment or Complex Environment Rearing Improves Spatial Learning and Reduces the Extent of Neuroplastic Activation during Memory Consolidation

Increased expression of basal polysialylated cell frequency may be suggestive of improved neuroplasticity. We evaluated this possibility by determining the magnitude of the learning-induced transient activation of NCAM PSA expression seen 12 h following training in both passive avoidance and water maze paradigms. Vehicle-treated animals exhibited learning-induced NCAM PSA activation following both tasks, the magnitude of which matched those observed previously (Fox *et al*, 1995; Murphy *et al*, 1996). Independent of task, animals receiving chronic nefiracetam, tacrine, or deprenyl exhibited no significant difference in the 12 h post-training frequency of NCAM PSA positive neurons as compared to the vehicle-treated control (Figure 4). However, for either nefiracetam, tacrine, or deprenyl treatment, a significant frequency increase over respective basal level was observed only in the avoidance conditioning paradigm and, proportionally, this was much reduced compared to that seen in the vehicle-treated group (Figure 4 and Table 2). Thus, drug-induced increase of basal polysialylated cell frequency markedly reduced the PSA activation required during consolidation of water maze or avoidance conditioning. By contrast, complex environment rearing resulted in a significant increase in the 12 h frequency following both avoidance conditioning and spatial learning paradigms although for both tasks the magnitude was reduced compared to social control animals (Figure 4 and Table 2). The alteration in degree of neuroplastic activation, as measured by NCAM PSA expression change, is most instructively viewed as percent learning-associated change with respect to the corresponding basal expression (Table 2).

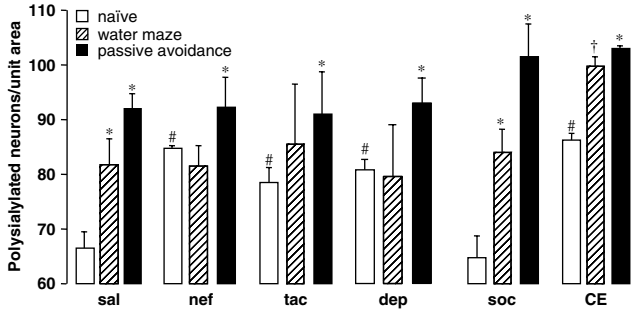

Figure 4 The influence of chronic treatment with tacrine (1 mg/kg/day), nefiracetam (15 mg/kg/day), deprenyl (5 mg/kg/day), and complex environment rearing on the increase in NCAM PSA-positive neurons following water maze or passive avoidance training. The data are mean ± SEM number of cells/0.15 mm^2 of the granule cell layer ($3 \leqslant n \leqslant 5$), values significantly different ($p < 0.05$; Student's *t*-test) from corresponding saline or social control are identified by a hash while trained values differing from the corresponding naïve animals are indicated by an asterisk. A cross indicates significant difference from both corresponding naïve animals and similarly trained, social control animals. sal: saline (0.9% w/v); nef: nefiracetam (15 mg/kg/day); tac: tacrine (1 mg/kg/day); dep: deprenyl (5 mg/kg/day); soc: social housing; CE: complex environment. NB: the last drug injection was administered 24 h prior to training to eliminate acute drug effects and animals reared in the complex environment were removed to social housing conditions 24 h prior to training.

The drug and environmental protocols employed here may also have improved neural robustness as it pertains to learning capacity, as demonstrated previously for the complex environment paradigm (Young *et al*, 1999). We analyzed the spatial learning ability of the animals reared in a complex environment and those that had received chronic treatment with nefiracetam, tacrine, or deprenyl but, importantly, were 24 h drug-free at the time of training. While two-way, repeated measures ANOVA did not detect an effect of drug treatment across all groups, it did reveal improved learning in the complex environment reared group compared to social controls ($F[3,16] = 1.6$, $p = 0.23$ and $F[1,8] = 7.29$, $p = 0.027$, respectively). However, both drug-treated and complex environment-reared animals exhibited significantly better performance across the five trials of the single water maze training session when compared with their corresponding controls (saline *vs* nefiracetam, tacrine, and deprenyl-treated, two-way ANOVA: $F[1,40] = 8.78$, $p = 0.005$; $F[1,40] = 5.73$, $p = 0.022$; and $F[1,40] = 5.78$, $p = 0.026$, respectively; social *vs* complex environment, two-way ANOVA: $F[1,40] = 19.08$, $p < 0.0001$) (Figure 5). It is noteworthy that with both nefiracetam and environmental interventions, animals exhibited significantly better performance even on the first water maze trial when these animals have no knowledge of an escape platform. This finding suggests that drug and environment can fundamentally alter behavior in a way that facilitates more efficient spatial learning. Given the robust nature of passive avoidance learning, it was not surprising that all groups, regardless of treatment or rearing environment, exhibited recall latencies that did not differ significantly from the criterion time of 600 s. Furthermore, neither treatment nor rearing environment altered recall of the avoidance task.

The relationship between enhanced basal hippocampal neuroplasticity and learning ability was further supported by the finding that these parameters were highly correlated. For each condition, the percent change in the basal NCAM PSA-positive cell number following the intervention (Table 2) was taken as an index of effect on neuroplasticity status while the mean latency to platform across all animals and all trials was taken as an index of spatial learning ability. Pearson analysis showed these measures of neuroplastic state and learning ability to be highly correlated

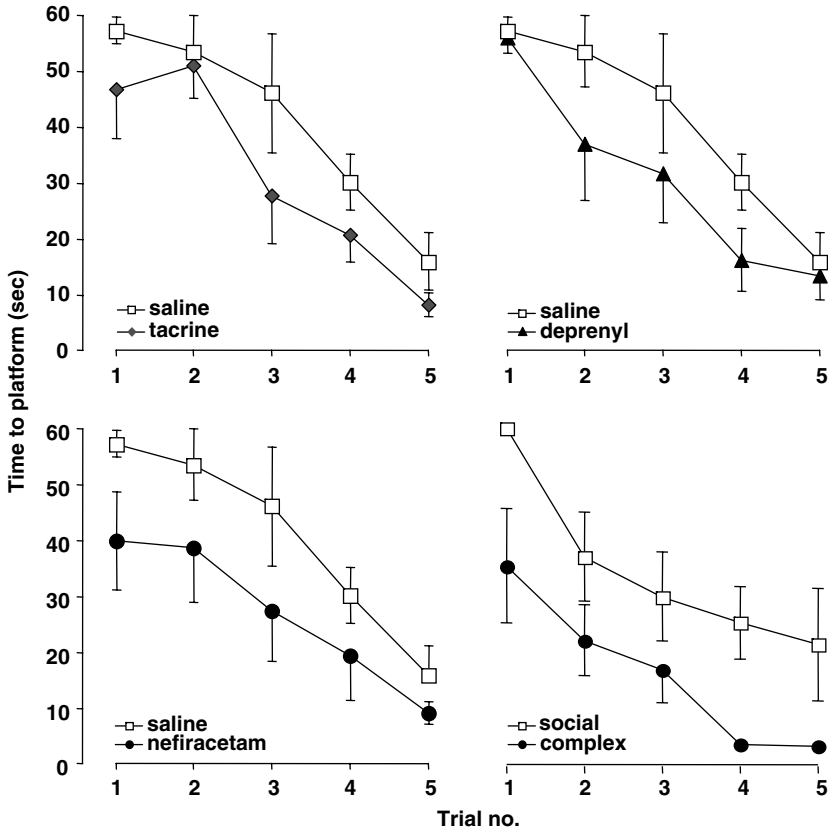

Figure 5 The consequence of chronic drug treatment and complex environment rearing on the spatial learning ability of rats. The figure shows the acquisition of water maze learning over five trials. Tacrine (1 mg/kg/day), deprenyl (5 mg/kg/day), nefiracetam (15 mg/kg/day), and complex rearing significantly improved acquisition of platform location (saline *vs* nefiracetam, tacrine, and deprenyl-treated, two-way ANOVA: $F[1,40] = 8.78$, $p = 0.005$; $F[1,40] = 5.73$, $p = 0.022$; and $F[1,40] = 5.78$, $p = 0.026$, respectively; social *vs* complex environment, two-way ANOVA: $F[1,40] = 19.08$, $p < 0.0001$). The data are the mean ± SEM time to reach the platform in seconds ($n = 5$). NB: the last drug injection was administered 24 h prior to training to eliminate acute drug effects and animals reared in the complex environment were removed to social housing conditions 24 h prior to training.

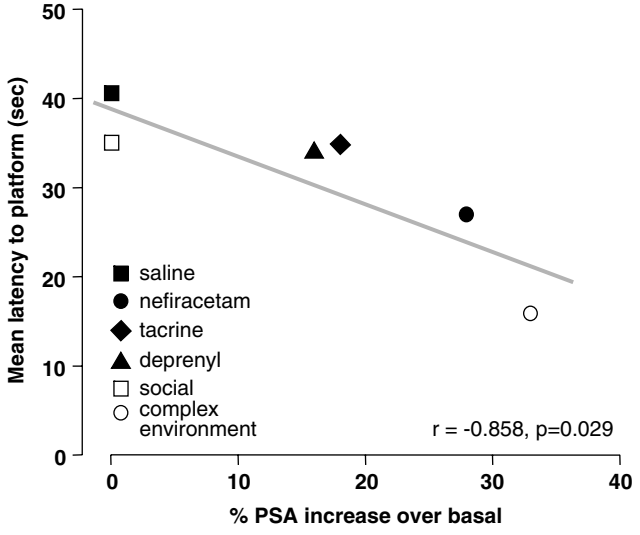

Figure 6 Relationship between hippocampal neuroplastic status and spatial learning ability. For each condition, the percent change in the basal NCAM PSA-positive cell number from the appropriate control group was taken from Table 2 as an index of effect on neuroplasticity status while the mean latency to platform across all animals and all trials was taken as an index of spatial learning ability. Pearson analysis showed these measures of neuroplastic state and learning ability to be highly correlated ($r = -0.858$, $p = 0.029$).

(Figure 6; $r = -0.858$, $p = 0.029$), indicating that the higher the basal NCAM PSA expression the better the learning ability of the animals.

Chronic Drug Treatment or Complex Environment Rearing Mediates Resistance to the Amnesic Action of Scopolamine

Here, the capacity of drug and environmental intervention to overcome a cholinergic deficit was investigated. In previous work we have shown that scopolamine administered 6 h following training induced robust amnesia for the passive avoidance task (Doyle and Regan, 1993). Here, both nefiracetam-treated and complex environment-reared groups showed a similar resilience to such scopolamine-induced amnesia of passive avoidance at the 24 h recall time (two-way ANOVA: $F[1,38] = 17.72$, $p = 0.0002$ and $F[1,21] = 13.3$, $p = 0.0015$, respectively) with an approximate 2.5-fold increase in the dose of scopolamine required to achieve an equivalent deficit to that observed in the control animals (Figure 7).

Combination of Drug and Environmental Interventions

If both cognition-enhancing drugs and complex environment rearing enhance neural robustness by similar

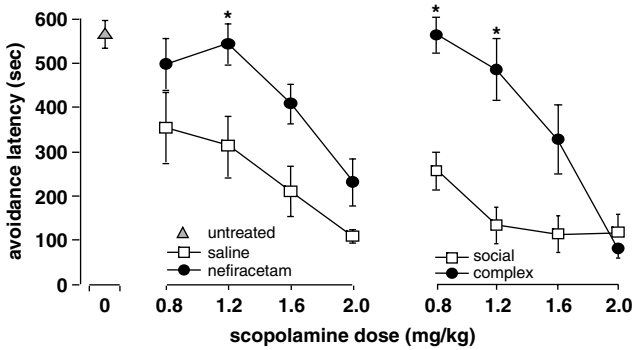

Figure 7 The consequence of chronic nefiracetam treatment and complex environment rearing on scopolamine-induced amnesia for the passive avoidance paradigm. The graphs show the resistance against scopolamine-induced amnesia (6 h post-training; i.p.) conferred by nefiracetam (15 mg/kg/day) and complex rearing (two-way ANOVA: $F[1,38] = 17.72$, $p = 0.0002$ and $F[1,21] = 13.3$, $p = 0.0015$, respectively). The data are the mean ± SEM of the escape latencies 24 h post-training ($3 \leqslant n \leqslant 8$). Values significantly different from the corresponding control are identified with an asterisk (Bonferroni *post hoc* test, $p < 0.05$). NB: the last injection of nefiracetam was administered 24 h prior to training to eliminate direct effects of the nootropic and animals reared in the complex environment were removed to social housing conditions 24 h prior to training.

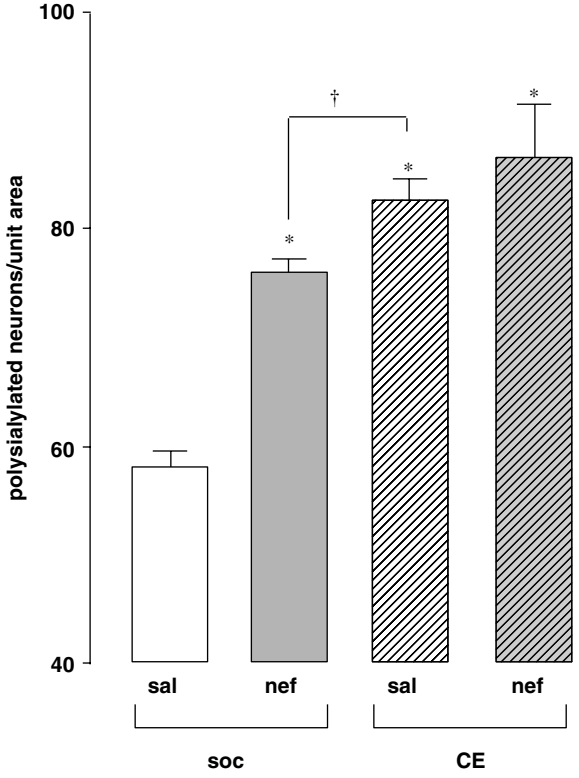

Figure 8 The influence of nefiracetam treatment and complex environment rearing alone and in combination on basal hippocampal NCAM PSA expression. Data represent mean ± SEM number of cells/0.15 mm^2 of the granule cell layer ($n = 4$). Significant difference from saline control and nefiracetam alone is indicated by an asterisk and cross, respectively (Student's *t*-test; $p < 0.05$). sal: saline (0.9% w/v); nef: nefiracetam (15 mg/kg/day); soc: social housing; CE: complex environment.

mechanisms then it may be expected that the system can be saturated. To address this, we examined the consequence of combining complex environment rearing and chronic treatment with a cognition-enhancing drug. While the enhancement of basal NCAM PSA-positive cell frequency following nefiracetam treatment alone was slightly less than that achieved by complex environment rearing, animals receiving both interventions exhibited no significant difference in dentate polysialylated cell frequency compared to either intervention alone (Figure 8).

Collectively, these data would tend to confirm the dominant effect of chronic drug treatment or complex environment rearing to be the development of a more robust neural structure.

DISCUSSION

Central to activity-related brain plasticity are enriched environments (Rosenweig *et al*, 1962). A great number of studies have demonstrated that environmental stimulation increases dendritic arborization and produces change at the level of the synapse in a manner that results in more robust cognitive function (Walsh *et al*, 1969; Greenough and Volkmar, 1973; Diamond *et al*, 1976; Moser *et al*, 1994; Nilsson *et al*, 1999). It is, therefore, not surprising that, in this study, complex environment rearing was found to enhance the frequency of polysialylated cells in the dentate infragranular zone and concomitantly improve spatial learning ability. Of particular interest, however, was the observation that chronic treatment with tacrine, nefiracetam, or deprenyl resulted in an increase in polysialylated cell frequency that was indistinguishable from that obtained with rearing in a complex environment.

Given that expression of polysialylated NCAM is a prerequisite for activity-dependent synaptic plasticity (Muller *et al*, 1996; Theodosis *et al*, 1999), it is perhaps not surprising that enhancing cholinergic function, long known to be necessary for memory consolidation (Doyle and Regan, 1993; Everitt and Robbins, 1997; Milner *et al*, 1998), modulates expression of this neuroplastic mechanism. Tacrine directly enhances acetylcholine transmission by inhibition of AChE while deprenyl will increase acetylcholine activity indirectly via improved dopamine function (Tariot *et al*, 1987; Piccinin *et al*, 1990; Mangoni *et al*, 1991; Molinengo and Ghi, 1997). Nefiracetam also exerts strong effects on cholinergic function and, *in vitro*, this nootrope has been shown to enhance neuritogenesis and NCAM PSA expression (Odumeru *et al*, 1997; Woodruff Pak and Hinchliffe, 1997; Oyaizu and Naharishi, 1999; Nishizaki *et al*, 2000; Narahashi *et al*, 2004). It is also likely that other transmitter systems enhancing neural activity will contribute to this neuroplastic response as previous studies have suggested activation of NMDA receptors to result in increased polysialylation in *ex vivo* preparations of neural tissue (Bouzioukh *et al*, 2001). However, drugs that dampen neural activity, such as NNC-711 and phenytoin, despite the cognition enhancing properties of the former, appear to have no effect on polysialylated cell frequency.

Thus, chronic treatment with a cholinergic activity-promoting, cognition enhancing drug or complex environment rearing increased the basal polysialylated cell

frequency of the hippocampal dentate. However, the consequence of such enhanced basal plasticity for memory consolidation-associated activation of neuroplastic mechanisms remained unclear. To address this issue we evaluated the consequence of these protocols on the transient change in polysialylated cell frequency seen 12 h following training in either an avoidance conditioning or spatial learning paradigm. The magnitude of the polysialylation activation is significantly reduced following either drug- or environment-mediated enhancement of basal PSA expression. Of importance in this regard are recent studies that have compared the transient polysialylation response in individual rats and shown the response to be greater in animals exhibiting greatest difficulty in task acquisition (Sandi *et al*, 2004), suggesting the need for a more substantial NCAM PSA response in the consolidation of complex/difficult tasks. An alternative explanation for reduced learning induced activations would be the existence of a ceiling or maximal NCAM PSA expression level at postnatal day 80. However, this is unlikely as, for example, water maze trained animals would then be expected to activate up to this 'ceiling', but these groups fail to exhibit any polysialylation increase at the 12 h time point following drug interventions.

Our observations are consistent with the idea that a chronic drug regimen or a complex environment induces a more robust memory pathway that can acquire and consolidate new information more efficiently. This hypothesis finds support in the improved learning behavior and enhanced resistance to cholinergic deficits seen following either intervention. Furthermore, the level of enhancement of basal neuroplastic status achieved by either drug or environmental intervention correlates directly with improved spatial learning ability. It is important to remember that improved learning and enhanced neuroplasticity result from alterations due to chronic exposure to these drugs and are not a consequence of acute drug action as drug treatment was ceased 24 h prior to training. Moreover, as a combination of both protocols failed to further increase basal polysialylated cell frequency, complex environment rearing and chronic drug regimens most likely attain a similar set point of PSA expression by the same mechanism(s).

As the frequency of the dentate polysialylated neurons declines markedly with age in both rodent and human populations (Fox *et al*, 1995; Abrous *et al*, 1997; Ní Dhúill *et al*, 1999), the effects of a chronic drug regimen or a complex environment may relate to an attenuation in the decline of this cell population. Indeed, polysialylated cell frequency declines earlier in animals reared in social groups as compared to a complex environment (unpublished observations). Such environmental or pharmacological intervention may prove highly relevant in certain disease states with well defined times of onset. For example, the expression of NCAM PSA is reduced in the hippocampal dentate of schizophrenic patients suggestive of a deficit in memory associated synaptic plasticity mechanisms (Barbeau *et al*, 1995). Thus, the benefits accruing from such procognitive interventions have the potential to oppose and even reverse aberrant synaptic connectivity patterning that likely underlie the developmental emergence of disorders such as autism, schizophrenia, and Alzheimer's disease (Braak and Braak, 1991; Bobinski *et al*, 1995; Scheff *et al*, 1996; Feinberg, 1982; Hoffman and McGlashan, 1997; Mirnics *et al*, 2001; Volkmar and Pauls, 2003).

These studies suggest that improved memory-associated synaptic plasticity may be the fundamental mechanism underlying the disease modifying action of drugs such as AChEIs (Farlow *et al*, 2000). In support of this, in Alzheimer's disease, for example, there is a striking up-regulation of NCAM polysialylation, but not NCAM polypeptide, in the dentate molecular layer (Gillian *et al*, 1994; Mikkonen *et al*, 1999). This most likely relates to repair associated with disappearance of degenerating axon terminals and reinnervation of the vacant postsynaptic sites with sprouted terminals of intact axons. This is consistent with the increased expression of NCAM PSA observed in the rat dentate molecular layer in response to the synaptic remodelling associated with lesion of the entorhinal inputs (Miller *et al*, 1994; Styren *et al*, 1994). Facilitation of synaptic reorganization, at least in part mediated by NCAM PSA, may represent an essential component of the purported disease-modifying actions of AChEIs. These studies suggest the molecular and cellular events underpinning neuroplastic responses to be novel targets for interventive drug strategies. Indeed, agents that more directly harness and enhance mechanisms of synaptic plasticity may be capable not only of symptomatic relief but reversal of fundamental deficits underpinning diseases such as Alzheimer's disease and schizophrenia.

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