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Anti-ischemic and cognition-enhancing properties of NNC-711, a γ-aminobutyric acid reuptake inhibitor

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Abstract

NNC-711 [1-(2-((diphenylmethylene)amino)oxy)ethyl)-1,2,4,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride], a γ-aminobutyric acid (GABA) reuptake inhibitor with anticonvulsant activity, was investigated with respect to its cognition-enhancing and neuroprotective potency. In the rat, administration of NNC-711 immediately prior to training prevented amnesia for a passive avoidance task induced by the acetylcholine receptor antagonist scopolamine. NNC-711 was also effective in protecting against ischemia-induced death of CA1 pyramidal neurons in a model of bilateral common carotid artery occlusion in the gerbil. In addition to a neuroprotective activity, NNC-711 exhibited significant cognition-enhancing actions. Daily administration of NNC-711, immediately prior to a spatial learning task, significantly reduced escape latencies in the water maze paradigm in both mature (postnatal day 80) and aged (28 months) rats. All of the above actions exhibited a bell-shaped response with an optimal dose of 0.5–1.0 mg/kg. These investigations with NNC-711 and previous clinical observations on the structurally related anticonvulsant tiagabine confirm the potential of GABA reuptake inhibitors as anti-amnesia and cognition-enhancing agents.

Keywords: GAT1 inhibitor; Tiagabine; Avoidance conditioning; Spatial learning; Carotid occlusion; CA1 cell death

1. Introduction

Reuptake of γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system (CNS), is the primary mechanism by which the action of synaptically released GABA is terminated. Specific GABA transporter (GAT) proteins that are electrogenic and sodium-dependent, and present on both neurons and glia, mediate this uptake (Kavanaugh et al., 1992; Malchow and Ripps, 1990). Pharmacological inhibition of this uptake mechanism may be beneficial in conditions where decreased GABAergic transmission has been implicated, most obviously in epilepsy. Indeed, tiagabine (NO-328 or (*R*)-1-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidinecarboxylic acid) and NNC-711 [1-(2-((diphenylmethylene)amino)oxy)ethyl)-1,2,4,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride] have proved to be effective anticonvulsants in animal models and, in the case of tiagabine, in the clinic (see Fig. 1; Suzdak et al., 1992; Borden et al., 1994; Smith et al., 1995). Four distinct GATs (GAT1–4) have been identified and cloned (Liu et al., 1993), and tiagabine and NNC-711 are both specific inhibitors of the GAT1 transporter (Borden et al., 1994).

In addition to their anticonvulsant activities, evidence from both in vivo and in vitro studies indicates GABAergic agents, including reuptake inhibitors, to possess neuroprotective and neurotrophic properties (Inglefield et al., 1995; Johansen and Diemer, 1991; Schuaib et al., 1994; Jolkkonen et al., 1996; Schwartz et al., 1995; Meier et al., 1984, 1987; Schousboe et al., 1985; Belhage et al., 1988). Specifically, in the post-ischemic period, tiagabine slows neuronal death in the gerbil hippocampus (Inglefield et al., 1995) and significantly reduces CA1 neuronal loss in the rat hippocampus (Johansen and Diemer, 1991). Furthermore, in the perforant pathway model of status epilepticus, tiagabine treatment reduces seizure-induced damage to hippocampal pyramidal cells as well as the impairment of spatial memory associated with this lesion (Halonen et al., 1996). There is also clinical evidence to suggest that GABA reuptake inhibitors, specifically tiagabine, improve performance in verbal memory and psychomotor speed

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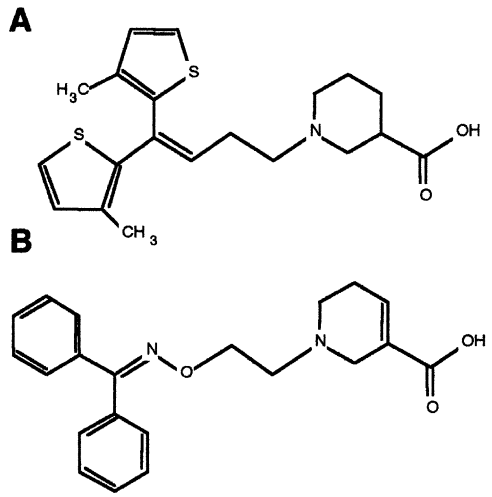

Fig. 1. Chemical structure of tiagabine (Panel A) and NNC-711 (Panel B).

when used as an anticonvulsant add-on therapy (Kalviainen, 1997).

Given the neuroprotective, anti-amnesic and cognition-enhancing activities attributed to tiagabine, this study aimed to determine if these actions could be attributed to GABA reuptake inhibitors in general using the NNC-711 analogue of tiagabine. The failure of more traditional pharmacological strategies to provide effective treatments for dementia and stroke provides the impetus for these investigations. Enhancing brain cholinergic function, via acetylcholinesterase inhibition, for example, has provided drugs such as tacrine, donepezil and rivastigmine, but with only limited cognition-enhancing potency (reviewed in Benzi and Moretti, 1998). In contrast, GABA is a major brain neurotransmitter whose broad inhibitory actions would oppose glutamate-mediated excitoxicity (reviewed in Green et al., 2000). Here, we have evaluated the ability of NNC-711 to protect against scopolamine-induced amnesia for the passive avoidance paradigm and also against ischemia-induced neuronal death in the gerbil hippocampus. Using a spatial learning paradigm, we also investigated the cognition-enhancing properties of NNC-711 in both mature and aged rats.

2. Materials and methods

2.1. Animal maintenance

Postnatal day 80 male Mongolian gerbils (50–65 g), postnatal day 80 male Wistar rats (300–350 g) and 28-month-old female Wistar rats were obtained from the Biomedical Facility, University College, Dublin. These were housed singly in a 12-h light/dark cycle with food and water available ad libitum. Animals employed for neurobehavioural studies were maintained and handled in the test environment for 3 days prior to the commencement of studies. All experimental procedures were approved by the Review Committee of the Biomedical Facility of University College, Dublin and were carried out by individuals holding the appropriate license issued by the Ministry of Health.

2.2. Drug administration

NNC-711 and scopolamine were administered by the intraperitoneal route in a final volume of saline that corresponded to 1 ml/kg for rats and 10 ml/kg for gerbils. For passive avoidance studies, NNC-711 was administered at the indicated dose at the 30-min pre-training time, while scopolamine (0.8 mg/kg) was administered at the 6-h post-training time, as has been described previously (Doyle and Regan, 1993). In water maze studies, NNC-711 was administered at the indicated dose 30 min prior to the first trial on each of the testing days but was not administered prior to the retention trial. To determine a neuroprotective effect on ischemia-induced CA1 cell death, NNC-711 was administered at the indicated dose just prior to carotid artery ligature.

2.3. Passive avoidance paradigm

The procedure was identical to that which we have described previously (Fox et al., 1995). Briefly, animals were trained in a one-trial, step-through, light–dark passive avoidance paradigm. The smaller, illuminated compartment was separated from a larger, dark compartment by a shutter, which contained a small entrance. The floor of the training apparatus consisted of a grid of stainless steel bars, which could deliver a remotely controlled, scrambled shock (0.75 mA every 0.5 ms; 2000 Hz) of 5-s duration when the animal entered the dark chamber. The animals were tested for recall of this inhibitory stimulus at 24-h post-training by placing them into the light compartment and noting their latency to enter the dark compartment. A criterion period of 300 s was used and values significantly different from the control were determined using the Mann–Whitney *U*-test for non-parametric data and P-values < 0.05 were considered to be significant. Immediately prior to training and recall, animals were placed in an open field apparatus and their spontaneous locomotion monitored.

2.4. Water maze paradigm

The paradigm employed was identical to that we have described previously (Murphy et al., 1996). The water maze apparatus consisted of a large circular pool (1-m diameter, 80 cm high, temperature 26 ± 1 °C) with a platform (11-cm diameter) submerged 1.5 cm below the

water surface. The water was 30 cm deep and the wall of the pool was 30 cm above the level of the water. A false bottom concealed the heating element and pump. Both the pool and the platform were constructed of black polyvinyl plastic and offered no intra-maze cues to guide escape behaviour. The experimental room contained several extra-maze visual cues. During testing, the platform was hidden in the same quadrant 30 cm from the sidewall. Each trial started with the rat facing the wall of the maze at one of three fixed points A, B, or C arranged triangularly around the pool (separated by 120°). The starting positions were used sequentially over the five trials of each training session. Continuity in the pattern of starting positions was maintained over different sessions, so that all starting points were used the same number of times. The time taken by the rat to find the hidden platform within a 60-s period was defined as the escape latency time. On the first trial, rats failing to find the platform within the 60-s period were placed on it for 10 s. Escape latencies were measured over five trials in each training session with an inter-trial test interval of 300 s. Training sessions were separated by a 24-h period, except for the retention test, which was performed 48 h following the preceding session. Visuomotor controls were required to locate a white platform raised above the water surface (2 cm) in the same quadrant used for the training trials. This visuomotor test was employed exactly as outlined previously for the trained group and was performed 72 h prior to the first training session, thereby, facilitating the identification of visually impaired animals.

2.5. Induction of transient global ischaemia and evaluation of associated neuronal damage

Transient global ischemia was induced in gerbils anaesthetized with 70 mg/kg sodium pentobarbitone, as we have described previously (Fox et al., 2001). Both the left and right common carotid arteries were exposed and blood flow occluded for 5 min by twisting with a small length of plastic tubing. The animals were allowed to recover and survive for 5 days before sacrifice. The brains were removed rapidly, coated in optimum cutting temperature compound and frozen using dry ice-cooled *n*-hexane. Frozen tissue sections (12 μm), stained with toluidine blue (1% (w/v) in 1% (w/v) sodium tetraborate), were used to determine the extent of ischemic damage in the CA1 region of the hippocampus. The sections were taken approximately midway along the septo-temporal axis (corresponding to −5.6 mm from Bregma in the rat, according to Paxinos and Watson, 1986). The total number of viable toluidine blue-stained cells remaining in the CA1 pyramidal cell layer was quantified in seven sections from each animal using procedures we have described previously (Fox et al., 2001). The sections were separated by 24 μm to preclude double counting of cells. The numbers obtained were divided by the length of the CA1 pyramidal cell layer, calculated from the end of the CA2 region to the point at which the CA1 pyramidal cells begin to disperse and form the subiculum, and normalised to a 400-μm length of the CA1 cell layer. The mean ± S.E.M. was established and these were used to establish the mean ± S.E.M. for each animal group ($n = 3$).

2.6. Statistical analysis

Statistical analysis employed the Mann–Whitney *U*-test and unpaired Student's *t*-test for the behavioural and ischemia studies, respectively. Statistical significance was determined using the INSTAT software programme (version 2.01) and *P*-values < 0.05 were considered to be significant. Where two-way and one-way analysis of variance (ANOVA) are also indicated, values were calculated using the PRISM software programme.

3. Results

3.1. Neuroprotective effects of NNC-711

The ability of NNC-711 to protect against scopolamine-induced amnesia was investigated using the rat pas-

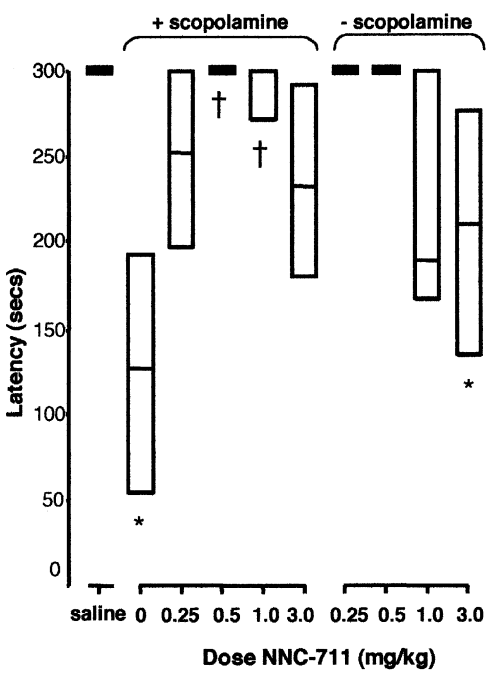

Fig. 2. Dose-dependent reversal of scopolamine-induced amnesia of avoidance learning by NNC-711. Values shown are the median and interquartile range of recall latencies at the 24-h post-training time ($n \geq 6$). NNC-711 was administered at the indicated doses 30 min prior to training and scopolamine (+scopolamine) or saline (−scopolamine) at a dose of 0.8 mg/kg at the 6-h post-training time. An asterisk indicates latency times significantly different from the saline control ($P < 0.05$). A cross indicates where NNC-711 significantly reversed scopolamine-induced amnesia as determined by latency times that differ significantly from the scopolamine-treated group that did not receive NNC-711 ($P < 0.05$).

sive avoidance paradigm. In agreement with previous studies (Doyle and Regan, 1993), administration of scopolamine at the 6-h post-training time produced a significant amnesia for the passive avoidance task when retrieval was tested at the 24-h post-training time, with no animals in the scopolamine-treated group achieving the 300-s criterion latency time (Fig. 2). Administration of NNC-711 during training significantly reversed the amnesia induced by scopolamine administration 6-h post-training when retrieval was tested at 24-h post-training, and this occurred within a dose range of 0.25–3.0 mg/kg (Fig. 2). The NNC-711 dose response appeared bell-shaped, with maximal effects occurring at 0.5–1.0 mg/kg and a reduced memory-sparing effect being observed at the 3 mg/kg dose. In the absence of scopolamine, NNC-711 had no effect on learning in the 0.25–0.5 mg/kg dose range, and induced amnesia at 3.0 mg/kg, although this was not as pronounced as that induced by scopolamine. Co-administration of NNC-711 (0.5–1.0 mg/kg) with scopolamine immediately prior to training produced no memory-sparing effects (data not shown). This suggested NNC-711 not to directly interact with the cholinergic system, but rather to have a more general neuroprotective action on the consolidation process.

The neuroprotective potency of NNC-711 was investigated further using the gerbil model of bilateral common carotid artery occlusion. Transient occlusion of carotid artery blood flow for a 5-min period produced a pronounced cell death in the hippocampal CA1 region 5 days following the procedure with a 33% loss of neurons observed compared to sham-operated animals (Fig. 3). Administration of NNC-711 immediately prior to occlusion significantly reduced CA1 cell death (Fig. 3). Complete protection against neuronal loss was observed at the lowest dose evaluated (0.5 mg/kg). The extent of the neuroprotective effect was reduced, although still significant, at a higher dose (3.0 mg/kg), indicating a similar bell-shaped response to that observed in the passive avoidance study. One-way analysis of variance also indicated a statistically significant difference between these four groups ($F = 11.746$; $P = 0.0027$; $df = 3$). Administration of NNC-711 to sham-operated control animals did not affect CA1 neuronal viability assessed at 5-day post-surgery (172.4 ± 14.5 cells/CA1 unit length, sham operated with no drug vs. 182.4 ± 1.0 cells/CA1 unit length, sham with 0.5 mg/kg NNC-711; $2 \leq n \leq 3$).

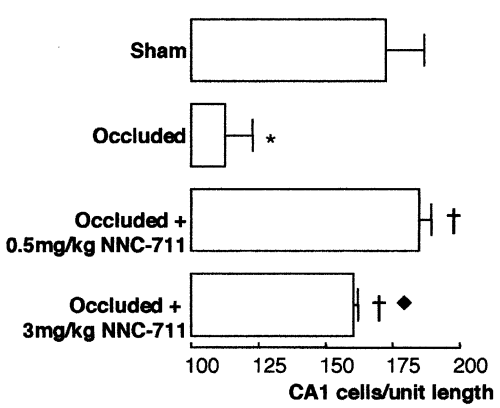

Fig. 3. Influence of NNC-711 on CA1 cell viability 5 days following bilateral common carotid artery occlusion. Sham-operated animals underwent identical surgical procedures except that the occlusion step was omitted. Where indicated, animals were administered NNC-711 via the intraperitoneal route immediately prior to carotid artery ligature. Values are the mean ± S.E.M. of viable toluidine blue-stained cells normalised to a 400-μm unit length of the CA1 cell layer. Values differing significantly from sham controls are indicated by an asterisk and those differing significantly from occluded animals that did not receive NNC-711 are indicated by a cross ($P < 0.05$; $n = 3$). A diamond indicates statistical significance between the two groups of occluded animals that received different doses of NNC-711.

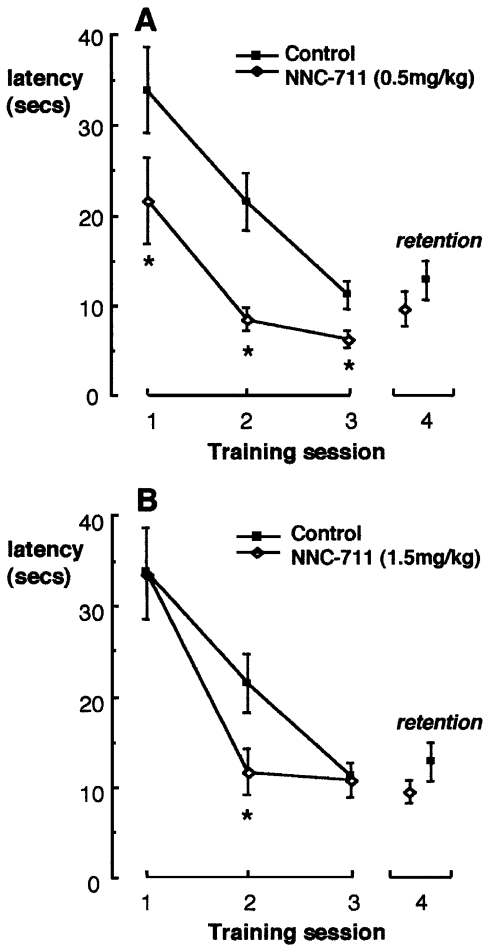

Fig. 4. Influence of NNC-711 on water maze learning in the mature (postnatal day 80) male rat. Values shown are the mean ± S.E.M. escape latencies on the final trial of the indicated training session and those differing significantly from saline-treated controls are indicated by an asterisk ($P < 0.05$; $n = 4$). The retention performance of the animals was assessed over another training session (session 4) 2 days following session 3. Animals received either saline (closed symbols) or NNC-711 (open symbols) at a dose of 0.5 mg/kg (Panel A) or 1.5 mg/kg (Panel B) 30 min prior to the commencement of each training session. For the retention test, however, the drug was not administered.

3.2. Cognition-enhancing effects of NNC-711

The cognition-enhancing actions of NNC-711 were evaluated by determining its influence on spatial learning ability in rats. Animals trained to navigate a water maze quickly learned to swim directly towards the hidden platform from any starting position at the circumference of the pool (Fig. 4A,B). Administration of NNC-711 (0.5 mg/kg) immediately prior to each training session significantly improved their ability to locate the hidden platform (Fig. 4A). A similarly significant but reduced effect was obtained at the higher concentration of 1.5 mg/kg, again suggesting the bell-shaped response typical of this GABA reuptake inhibitor (Fig. 4B). Two-way ANOVA confirmed the effect of NNC-711 on spatial learning, as a significant degree of variance was detected both between the control and drug treated groups ($P = 0.0188$; $F = 4.446$; $df = 2$) and across the different sessions ($P = 0.0022$; $F = 5.888$; $df = 3$). The interaction term was non-significant, however, with a probability of $P = 0.3539$ and $F = 1.151$ ($df = 6$). NNC-711 had no effect on memory retention, as this was similar in both treated and control groups when evaluated at 2 days following the third training session (Fig. 4A,B).

Similar cognition-enhancing effects were obtained with aged (28 months) female rats. These animals exhibited the expected age-related deficits in learning when compared to postnatal day 80 adult animals (escape latencies: postnatal day 80 control 11.2 ± 1.5 s vs. aged control 26.83 ± 4.14 s at third training session, $P < 0.05$; see also Figs. 4 and 5). Unlike the mature animals, in aged animals, NNC-711 was without effect on escape latencies in the last trial of the first water maze session (Fig. 5A). Thereafter, a marked decrease in escape latency was noted in the second and third training sessions, the former being significantly different from the control group of animals using the Mann–Whitney test (Fig. 5A). However, two-way ANOVA on these control and drug-treated groups over the three sessions returned non-significant results ($P = 0.1253$; $F = 0.2071$; $df = 1$). While this indicated the overall learning trend to be similar in both groups, the performance of drug-treated animals was significantly superior in the second session. Neither the learning deficits observed in the aged rat, nor their amelioration following NNC-711 administration, were attributable to visual or motor impairments, as no between-group differences were noted in ability to identify and swim to a platform exposed above the water (Fig. 5B) and this was confirmed by one-way ANOVA ($P = 0.61$; $F = 0.5162$; $df = 2$).

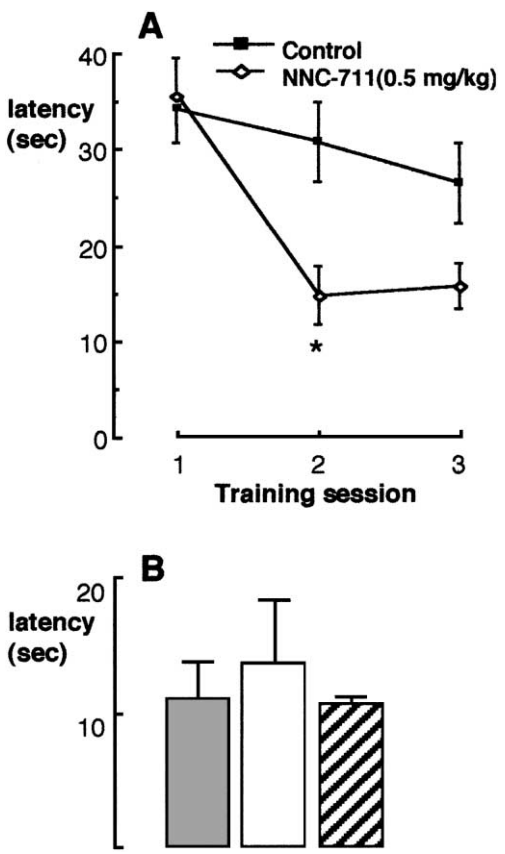

Fig. 5. Influence of NNC-711 on water maze learning and visuomotor skill in the aged (28 months) female rat. In Panel A, values shown are the mean ± S.E.M. escape latencies on the final trial of the indicated training session and those differing significantly from saline-treated controls are indicated by an asterisk ($P < 0.05$; $n = 5/6$). Animals received either saline (closed symbols) or NNC-711 (open symbols) at a dose of 0.5 mg/kg 30 min prior to the commencement of each training session. The influence of age and NNC-711 on visuomotor behaviour in the water maze is indicated in Panel B. Values shown are the mean ± S.E.M. of time taken to reach a visible platform for mature rats (grey bar), aged rats (white bar) or aged rats treated with 0.5 mg/kg NNC-711 30 min prior to the test (hatched bar).

4. Discussion

The present study demonstrates NNC-711, a GABA reuptake inhibitor, to be neuroprotective and to have cognition-enhancing activity. NNC-711 was found to protect against hippocampal pyramidal cell loss following ischemic insult in the gerbil and to reduce escape latencies in the water maze task in both young and aged rats. These results confirm the neuroprotective and cognition-enhancing properties of GABA reuptake inhibitors, and also support the ability of tiagabine to protect against ischemia-induced hippocampal damage in rodents (Inglefield et al., 1995; Johansen and Diemer, 1991) and improved cognitive skills observed in clinical trials (Kalviainen, 1997).

Tiagabine and NNC-711 appear to have virtually indistinguishable mechanisms of action. In synaptosomal membranes, they inhibit GABA uptake and in cultured cell lines, these drugs are twice as potent at inhibiting glial compared to neuronal GABA uptake, without having significant affinity ($IC_{50} > 10{,}000$ nM) for the dopamine, norepinephrine, serotonin, glutamate or choline uptake sites

(Suzdak et al., 1992). The resultant increase in synaptic GABA is thought to account for their anticonvulsant activity. Moreover, tiagabine and NNC-711 act specifically on the GAT transporters and are without significant affinity ($IC_{50} > 10{,}000$ nM) for the dopamine, noradrenaline, acetylcholine, adenosine, serotonin, histamine, opiate, glycine, glutamate, GABA, or sigma opioid receptors (Suzdak et al., 1992). However, the mechanism by which these drugs serve to inhibit GABA reuptake is unclear, as radiolabelled tiagabine is not a substrate for the carrier process nor does it alone alter the release of GABA from presynaptic neurons (Ostergaard et al., 1995).

NNC-711 was also found to be effective in reversing scopolamine-induced amnesia for a passive avoidance task in the rat. However, the same effect was not observed when NNC-711 was co-administered with the amnesic agent, implying that its memory-sparing function is not due to a direct cholinergic action. This is supported by the ligand-binding studies described above in which NNC-711 failed to exhibit any affinity for cholinergic receptors or choline uptake mechanisms (Suzdak et al., 1992). Rather, it would appear that NNC-711 has a more general memory-enhancing function that may be related to its neuroprotective mechanism of action. Conceivably, this neuroprotective potency may arise from restoration of the GABA-glutamate balance during situations of excitotoxicity. Indeed, several anticonvulsant drugs with GABAergic pharmacological profiles confer in vitro survival of hippocampal neurons exposed to neurotoxic levels of glutamate or extracellular potassium (Mattson and Kater, 1989). Moreover, since GABA receptor ligands and GABA itself exhibit neurotrophic properties in vitro and in vivo (Schousboe et al., 1985; Meier et al., 1984, 1987), agents potentiating the action of GABA would be expected to have similar effects. Other cognition-enhancing agents, notably the nootropic drug nefiracetam and the peptidergic drug cerebrolysin, are also neurotrophic, suggesting that this property may be predictive for cognition-enhancing/memory-sparing activity (Akai et al., 1992; Francis-Turner and Valouskova, 1996; Odumeru et al., 1997). Furthermore, since the neurotrophic action of GABA is developmentally regulated, it is plausible that in the adult brain, this effect is restricted to discrete populations of neurons that retain neurodevelopmental properties (Hansen et al., 1988; Belhage et al., 1988). For example, neuronal populations in which expression of polysialylated forms of the neural cell adhesion molecule (NCAM PSA)—a cellular correlate of neuroplasticity—persists into adulthood, are intimately involved in memory consolidation (Fox et al., 1995; Murphy et al., 1996; O'Connell et al., 1997).

Importantly, the dose range in which NNC-711 was most effective at enhancing cognition, 0.5–1.0 mg/kg, appears to be of clinical relevance as it concurs with the effective anticonvulsant doses of tiagabine (16–56 mg daily; reviewed in Leach and Brodie, 1998). This further supports the view that the cognition-enhancing potency of NNC-711 is directly related to a positive GABAergic function. Furthermore, the time-course for cognition enhancement by NNC-711 is in agreement with the elimination half-life of 7–9 h and peak plasma concentration time of 30–90 min observed for tiagabine. The neuroprotective and cognition-enhancing effects of NNC-711 exhibited bell-shaped dose–response effects. Higher doses of NNC-711 were less effective at protecting against ischemia-induced neuronal loss and scopolamine-induced amnesia. Moreover, in the absence of scopolamine, higher doses of NNC-711 impaired retention of the passive avoidance response.

Although the bell-shaped dose response is a feature of many cognition-enhancing drugs, such as the nootropes (Toide, 1989; Nabeshima, 1994), how this may relate to the mechanism of NNC-711 action is unclear. One possibility relates to observations that GAT transporters can operate in reverse, depending on which condition is most thermodynamically favourable (Cammack et al., 1994). This phenomenon is thought to account for Na^+-dependent non-vesicular release of GABA that occurs during high frequency neuronal firing and during seizure activity (Taylor and Gordon-Weekes, 1991). Reverse operation of the GAT1 transporter can result in the release of sufficient amounts of GABA to mediate activation of $GABA_A$ receptors (Gaspary et al., 1998). Given these opposing activities, GAT inhibitors tend to exhibit contrary effects. For example, in audiogenic seizure-prone rats, NNC-711 exerts an anticonvulsant action at lower doses but a proconvulsant action at higher doses (Smith et al., 1995). Furthermore, in a plus maze task, NNC-711 exhibited anxiolytic properties at low doses but disrupted behaviour at higher doses (Dalvi and Rodgers, 1996).

In the rational design of novel anti-epileptic drugs, the most successful approach has been the enhancement of GABA-mediated synaptic transmission via several distinct targets. Older GABAergic anticonvulsant drugs such as the benzodiazapines and barbiturates appear to bind directly to $GABA_A$ receptor subunits and potentiate the action of GABA via enhanced Cl^- channel functioning. However, these have adverse effects on cognitive function and are known to impair memory, attention and psychomotor speed in animals and humans (reviewed in Costa and Guidotti, 1996). Newer GABAergic agents, however, do not appear to cause cognitive deficits and increasing evidence suggests that these may facilitate or enhance cognitive function. For example, vigabatrin, a selective reversible inhibitor of GABA transaminase, is known to significantly improve both episodic and semantic memory, as well as concentration and flexibility of mental processing (Kalviainen et al., 1991, 1995). Similarly, in add-on studies with gabapentin, which is thought to increase GABA levels via enhanced synthesis, improved performance on measures of memory and attention were observed (Mortimore et al., 1998; Meador et al., 1999). As yet, it is unclear why GABAergic agents affect cognition differ-

ently; nevertheless, their potential as cognition-enhancers remains unexplored, as this aspect of their function has only been serendipitously uncovered in studies directed to the detection of adverse cognitive effects during anticonvulsant therapy.

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