Nefiracetam Prevents Propofol-induced Anterograde and Retrograde Amnesia in the Rodent without Compromising Quality of Anesthesia

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Background: Propofol is a short-acting intravenous anesthetic agent. However, cognitive function remains depressed for several hours thereafter. We have evaluated the ability of nefiracetam, a novel cognition-enhancing agent, to alleviate propofol-induced amnesia in a rodent model of learning.

Methods: Rats were trained in a one-trial, step-through, light-dark passive avoidance paradigm. Propofol (10 and 75 mg/kg) was administered by the intraperitoneal route at 15 min before training and separately at increasing times in the immediate 0–6 h post-training period (100 and 150 mg/kg). Nefiracetam, 9 mg/kg, was administered by the intraperitoneal route 1 h before training. Animals were tested for recall at the 12 h post-training time, and after their killing, immunocytochemistry was used to determine the increase in hippocampal neuronal polysialylation, an event associated with memory consolidation. Induction and duration of anesthesia induced by propofol was determined using tail pinch and pedal withdrawal reflexes.

Results: Propofol-induced anterograde amnesia occurred in a dose-dependent manner. Induction of retrograde amnesia required a higher dose of propofol, which anesthetized the animals and was effective only in the immediate 3-h post-training period. In the absence of any evident effect on the onset or duration of anesthesia, nefiracetam prevented both forms of propofol-induced amnesia and preserved the learning-associated changes of neuronal polysialylation state.

Conclusions: The ability of nefiracetam to prevent propofol-induced anterograde and retrograde amnesia is proposed to be indirect and to result from modulation of gene transcription in a manner that initiates a cascade of events involving protein synthesis leading to synaptic growth associated with the formation of the long-term memory trace. (Key words: Cognition enhancement; neural cell adhesion molecule; passive avoidance paradigm; polysialylation.)

PROPOFOL (disopropylphenol; Diprivan) is a popular short-acting intravenous anesthetic agent.1–6 To date little is known about the effects of propofol on the molecular aspects of memory formation. In rodents, subanesthetic doses of propofol produce anterograde amnesia of an avoidance task but fail to elicit retrograde amnesia in a similar concentration range.7 In humans propofol produces anterograde amnesia for intraoperative events.8 However, there is still debate as to whether implicit memory may be affected.9–11 In addition, cognitive function associated with learning, language, reasoning, and planning remain depressed for several hours after cessation of propofol administration.12 Despite the clinical and financial implications, few agents are known to facilitate recovery from the cognitive deficits associated with propofol-induced anesthesia.13–16

Nefiracetam, a pyrrolidine derivative, is a recently developed cognition-enhancing agent that preserves memory formation in a variety of animal models with compromised learning.17 The effects of these agents emerge gradually and manifest within a 16- to 24-h period after training.18 As these agents are ineffective in adrenalectomized animals, it has been suggested that, in the absence of corticosteroid production, they cannot modulate gene transcription in the manner that initiates a cascade of events involving protein synthesis and leads to the synaptic growth associated with the formation of the long-term memory trace.19 For example, pretraining administration of nefiracetam prevents scopolamine-
compromised learning and an associated modulation of neural cell adhesion molecule (NCAM) polysialylation state, a neuroplastic marker, in a late phase of memory consolidation during which effective concentrations of the drug would no longer be available. Such modulations have been attributed to the increased frequency of polysialylated granule cell-like neurons at the hilar border of the granule cell layer of the adult rat dentate gyrus in the 10- to 12-h post-training period. Enduring effects of transient nefiracetam exposure have been observed in vitro using the PC-12 pheochromocytoma cell model. Preincubation of these cells with nefiracetam subsequently results in a significant enhancement of NCAM polysialylation state and associated neuritogenesis after exposure to nerve growth factor alone. Thus nefiracetam increases long-term, post-training modulations of neuroplastic events, which are likely to contribute to the synaptic rearrangements that underlie memory consolidation.

The present studies have investigated modulations of NCAM polysialylation in combination with recall of a passive avoidance paradigm to determine the influence of subanesthetic and anesthetic doses of propofol on anterograde and retrograde amnesia in the rat. In addition, we have evaluated the ability of coadministered nefiracetam on these two parameters to determine its potential use in attenuating propofol-induced amnesia.

**Methods and Materials**

**Passive Avoidance Training**

Rodents innately avoid direct light and, when provided with the option, consistently tend to select darker areas. This provides the basis for the step-through, light-dark passive avoidance response that we have used in the present and previous studies. Postnatal day 80 male Wistar rats (300–350 g) were used. These were housed singly under standard conditions, with food and water available ad libitum. Animals were introduced, maintained, and handled in the test environment for 3 days before commencement of studies. In this classical conditioning task, the unconditioned stimulus is provided by placing the animal in an illuminated environment with the option of escaping into a darkened chamber. Herein the conditioning stimulus (a mild foot shock of 0.75 mA for 5 s) was given, thus allowing an association between the unconditioned and conditioned stimulus to be learned. The animals were tested for recall 12 h post-training by placing them into the light chamber and recording their latency to enter the dark compartment. This was used as a measure of their ability to remember the aversive stimulus. A criterion period of 600 s was used, and this was assigned to all animals that did not enter the darkened compartment within this time. Immediately before training and recall animals were exposed to an open-field apparatus (62 cm × 62 cm × 15 cm). The walls were black and the white floor marked into an 8 × 8 grid. The number of lines crossed in a 5-min period was used as a measure of locomotor activity.

**Drug Administration**

Anterograde amnesia was investigated by administering propofol (10 and 75 mg/kg; Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK) by the intraperitoneal route in 0.9% saline containing 10% Tween-20 (Sigma Chemical Co. Ltd., Poole, Dorset, UK) 15 min before training. In a separate series of experiments on retrograde amnesia, propofol (100 and 150 mg/kg) was administered to individual groups at discrete post-training time points of 0, 1, 2, 3, 4, 5, and 6 h. An equal volume of 0.9% saline containing 10% Tween-20 served as a control. When nefiracetam (9 mg/kg; Daichi Pharmaceutical Company Co. Ltd., Tokyo, Japan) was used to attenuate the amnesia induced by propofol, it was administered by the intraperitoneal route in 0.9% saline 1 h before training. An equal volume of 0.9% saline served as a control. After this, the experimental design protocol comprised four groups—a control group receiving saline and saline/Tween-20; a propofol-treated group receiving propofol and saline; nefiracetam-treated group receiving nefiracetam and saline/Tween-20; and a propofol and nefiracetam-treated group receiving propofol and nefiracetam. The period required for propofol-induced anesthesia was determined by measuring loss of a tail tip pinch and pedal withdrawal reflexes after a pinch to the digits. The duration of propofol-induced anesthesia was determined by return of the righting reflex.

All experimental procedures were approved by the Review Committee of the Biomedical Facility of University College, Dublin, and were carried out by people holding the appropriate license issued by the Irish Minister of Health.

**Quantification of Hippocampal Polysialylated Neurons**

All animals were killed by cervical dislocation followed by decapitation to facilitate removal of the brain. This was
NEFRACETAM PREVENTS PROPOFOL-INDUCED AMNESIA

rapidly coated in Optimal Cutting Temperature (OCT®, Agar, Stansted, Essex, UK) compound, snap frozen in dry ice-cooled n-hexane, and stored at −80°C until required for further processing. Polysialic acid (PSA) immunocytochemistry was used to detect hippocampal polysialylated neurons using techniques described previously.22 Cryostat axial sections of 12 μm were fixed in 70% (v/v) ethanol and incubated overnight with anti-PSA ascitic fluid diluted 1:500 (generous gift of Prof. G. Rougon23). The sections were then exposed for 3 h to fluorescein-conjugated goat anti-mouse IgM diluted 1:100 (Calbiochem, Beeston, Nottingham, UK) and mounted in Citifluor® (Agar, UK), a fluorescence-enhancing medium. Where relevant, nuclei were fluorescently, counter-stained by a brief exposure (60 s) to propidium iodide (40 ng/ml PBS, Sigma Chemical Co., UK). The number of PSA-immunopositive neurons at the granule cell layer and hilus border were counted in 10 alternate 12-μm sections commencing at −5.6 mm with respect to Bregma, to preclude double counting of the 5–10 μm perikarya. Cell counts were divided by the total area of the granule cell layer and multiplied by the average granular cell layer area, which is 0.15 ± 0.01 mm² at this level, the mean ± SEM calculated, and the results expressed as numbers of PSA-positive cells per unit area. Area measurements were performed using a Quantimet 500 Image Analysis System.

Statistical Analysis
All behavioral data are expressed as the median and interquartile range, and their statistical significance was

Fig. 1. Propofol-induced anterograde and retrograde amnesia of a passive avoidance response in the rat. The effects of propofol, administered 15 min before training or 3 h after training, on recall at the 12 h post-training time are illustrated. Latencies represent the median and interquartile range (n = 6) and those significantly different (P < 0.05; Mann-Whitney U-test) from control animals (open columns) are indicated by an asterisk.

Fig. 2. Time-dependent nature of propofol-induced retrograde amnesia. Propofol, 150 mg/kg, was administered to separate groups at increasing times after training, and recall was assessed at the 12-h post-training time. Values represent the median and interquartile range (n = 6) and those significantly different (P < 0.05; Mann-Whitney U-test) from control animals are indicated by an asterisk.

determined initially using Kruskal-Wallis non-parametric analysis of variance (ANOVA) followed by the Mann-Whitney non-parametric U test. The immunohistochemical data are presented as mean ± SEM with statistical significance determined initially using ANOVA followed by the Student’s t test. In both cases, the control group was compared separately with each treatment group, and values of P < 0.05 were accepted to be significant.

Results
All control animals readily learned the task as judged by their increased latencies to enter the darkened chamber at 12 h post-training time. In contrast, those treated with 75 mg/kg propofol were amnesic as evidenced by their reduced latencies (KW = 9.33; P = 0.0023; fig. 1). This effect was dose-dependent as it was not observed at the lower dose of 10 mg/kg. The amnesia induced by 75 mg/kg propofol was not attributable to behavioral anomalies as there was no difference in locomotor activity or exploratory behavior between control and propofol-treated animals 5 min before training (144.0 ± 6.5 vs. 135.0 ± 24.2 lines crossed/5 min in treated and control animals, respectively) or just before the 12 h recall time (110.6 ± 4.9 vs. 119.3 ± 13.0 lines crossed/5 min in treated and control animals, respectively). In addition, during training the latencies to enter the darkened chamber were not significantly different between control animals and those treated with nefiracetam, nefiracetam.
with propofol, or propofol alone (24.3 ± 8.1 vs. 33.3 ± 3.2, 19.0 ± 4.0, or 30.0 ± 3.2, respectively).

The induction of retrograde amnesia in the post-training period required substantially larger doses of propofol, which resulted in a complete loss of consciousness. Animals treated with 150 mg/kg, but not 100 mg/kg, at 3 h after training failed to recall the task at the 12 h post-training time (KW = 7.62; P = 0.0038; fig. 1) despite full recovery of consciousness as evidenced by the lack of open-field behavioral anomalies (158.0 ± 25.8 vs. 165.0 ± 13.5 lines crossed/5 min in treated and control animals, respectively). The induction of retrograde amnesia by propofol was time-dependent as administration of effective anesthetic concentrations at post-training times in excess of 3 h did not result in recall failure (KW = 11.65; P = 0.003; fig. 2). Furthermore, no behavioral anomalies were observed at the 12 h recall time in animals that had received 150 mg/kg propofol in the 3 h post-training time (88.7 ± 9.4 vs. 92.3 ± 13.1 lines crossed/5 min in treated animals and those receiving vehicle alone, respectively).

The retrograde amnesia induced by a 15-min pretraining dose of propofol, 75 mg/kg, could be completely prevented by administration of nefiracetam, 9 mg/kg, 1 h before training (KW = 5.61; P = 0.029; fig. 3A). Animals receiving a combination of propofol and nefiracetam or nefiracetam alone exhibited recall latencies that were indistinguishable from those observed in the trained control group. Similar results were obtained for retrograde amnesia induced by higher doses of propofol, 150 mg/kg (KW = 13.52; P = 0.0036; fig. 4A). Nefiracetam had no effect on the onset or duration of propofol-induced anesthesia in this group of animals. A similar induction period was observed in those treated with propofol alone and in combination with nefiracetam with respect to loss of tail pinch and pedal withdrawal reflexes (199.0 ± 6.2 vs. 181.0 ± 14.1 s for propofol alone at 3 h post-training and with nefiracetam given 1 h
NEFIRACETAM PREVENTS PROPOFOL-INDUCED AMNESIA

Fig. 4. Influence of nefracetam on task recall and NCAM polysialylation state after propofol-induced retrograde amnesia. In Panel A, recall is compared between control animals and those treated with propofol alone (150 mg/kg at 5-h post-training) and in combination with nefracetam (9 mg/kg at 1 h pretraining). Nefracetam alone had no effect on recall. The frequency of polysialylated neurons in these groups is shown in Panel B. Treatment with propofol significantly attenuated both parameters (P < 0.05; Mann-Whitney U-test and Student's t test for panel A and B respectively) as indicated by %. Similarly, both were significantly preserved by administration of nefracetam with propofol compared with animals that received propofol alone as indicated with an asterisk. Values represent the median and interquartile range and the mean ± SEM in panels A and B respectively (n = 6). C = control; P = propofol; P + N = propofol and nefracetam; N = nefracetam.

The ability of nefracetam to prevent propofol-induced anterograde amnesia was also apparent with respect to the learning-associated modulations of neuronal polysialylation state in the dentate gyrus of the hippocampal formation. As demonstrated previously,21,22 animals trained in a passive avoidance response exhibit a significant increase in polysialylated cell number in the 12-h post-training period (df = 23; F = 5.43; P = 0.0043; figs. 5B and 5). In animals rendered amnesic by pretraining administration of propofol, 75 mg/kg, the number of polysialylated neurons was indistinguishable to those observed immediately after training or in the naive, untrained animal. In contrast, animals in which propofol-induced anterograde amnesia had been prevented by administration of nefracetam, 1 h before training, exhibited the same increase in polysialylated neurons to that observed in the trained control group. In the same manner, nefracetam prevented propofol-induced retrograde amnesia and preserved the learning-associated increase in polysialylated dentate neurons within the hippocampal formation (figs. 4B and 6). This could not be attributed to nefracetam increasing the basal expression of these polysialylated neurons as there was no significant difference in PSA-positive dentate neurons in untrained animals 1 h after administration of 9 mg/kg nefracetam as compared with vehicle-treated controls (69 ± 3 vs. 64.5 ± 2.3, respectively). Furthermore, previous studies have excluded an effect of nefracetam alone on the training-induced modulations of NCAM polysialylation.20

Discussion

The anesthetic action of propofol demonstrates two unique aspects relating to the conversion of short-term
memory to long-term storage. The relatively low concentrations required to produce anterograde amnesia in comparison to the twofold dose increase necessary for retrograde amnesia suggests the memory trace to be initially labile but to become more robust with time. Second, the induction of retrograde amnesia could only be produced within the first 3 h post-training period, indicating that thereafter memory is committed to a process that eventually results in long-term storage. This period immediately precedes that in which increased spin frequency is observed after passive avoidance learning. In a previous study, Pang et al. demonstrated propofol to induce anterograde amnesia in mice in an identical dose-dependent manner. However, they failed to observe retrograde amnesia as the maximal dose administered during the post-training period was 100 mg/kg, at which we also failed to induce amnesia. The induction of anterograde amnesia within this dose range has an obvious clinical advantage in the prevention of recall for perioperative events that may occur because of inadequate anesthesia.

The effectiveness of nefiracetam in preventing the anterograde and retrograde amnesia induced by propofol has been demonstrated by two separate criteria—task recall and preservation of learning-associated modulations of NCAM polysialylation state. In the case of anterograde amnesia, the animals clearly acquired and retained the task as they had total recall in the presence of nefiracetam. This memory-sparing effect cannot be direct as nefiracetam alone did not significantly improve learning in the task used; however, this point may be considered equivocal as the recall latencies measured were close to criterion. Furthermore, nefiracetam alone had no effect on task-associated modulation of NCAM polysialylation. The memory-sparing action of nefiracetam is unlikely to be specific to amnesia produced by propofol as previous studies have shown precisely the same effects on recall and modulation of NCAM polysialylation in situations of scopolamine- and apomorphine-induced anterograde and retrograde amnesia in rodents. It is unlikely that this action is specific to a transmitter system as nefiracetam, and nootropes in gen-

Fig. 5. Frequency of PSA-positive dentate neurons after propofol-induced anterograde amnesia. Panels A and B are representative of the immunoreactivity pattern observed in trained animals at the 0-h and 12-h post-training times, respectively. The frequency of immunopositive neurons is significantly reduced in the propofol-treated group (Panel C) but preserved by coadministration of nefiracetam (Panel D) compared with that observed at 12 h in the trained animal (Panel B). H = hilus; GCL = granule cell layer; GCL/H = granule cell layer hilar border.
Fig. 6. Frequency of PSA-positive dentate neurons after propofol-induced retrograde amnesia. Panels A and B are representative of the immunoreactivity pattern observed in trained animals at the 0-h and 12-h post-training times, respectively. The frequency of immunopositive neurons is significantly reduced in the propofol-treated group (Panel C) but preserved by coadministration of nefiracetam (Panel D) compared with that observed at 12 h in the trained animal (Panel B). H = hilus; GCL = granule cell layer; GCL/H = granule cell layer hilar border.

General are known to activate cholinergic, dopaminergic, and glutamatergic function. Although it cannot be discounted, it is unlikely that the affinity of nefiracetam for GABA_A receptors counteracts the proposed inhibitory action of propofol on this transmitter system because the quality of anesthesia is unaffected in the presence of nefiracetam. Previous studies have suggested modulation of the corticosteroid axis to be a potential cognition-enhancing action of nootropic agents as they failed to elicit such enhancement in adrenalectomized animals, in which corticosteroid modulation of gene transcription events is compromised. Thus, it is likely that nootropes increase early events in memory formation rather than by enhancing cognition per se. This would be consistent with the late emergence of their memory-enhancing effects. However, the latter studies are not directly comparable with the results of the present investigation in which the anti-amnesic action of nefiracetam was evaluated in propofol-compromised memory formation. As a consequence the earlier emergence of this action may reflect a more immediate effect of this agent on compromised learning, an effect that requires further investigation in periods that precede the 12-h post-training time.

Should these findings translate to clinical practice, their relevance would relate mainly to the use of nefiracetam in the reversal of anterograde amnesia. In ambulatory anesthesia, which accounts for up to 60-70% of all surgery, there is a need to ensure rapid recovery for patient security and economy of health care. Doxapram, aminophylline, and flumazenil have been investigated with a view to alleviating these effects. However, these have not experienced widespread use presumably because of their short duration of action. Preservation of memory for events before induction of anesthesia is attractive. However, administration of nefiracetam preoperatively would be ill-advised until the influence of this agent on perioperative memory processing has been evaluated.

References


