# Effects of chronic buproprion and nicotine administration on cell genesis and DNA fragmentation in adult rat dentate gyrus

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## Abstract

Previous experiments have shown that chronic subcutaneous administration of nicotine dose-dependently inhibits the acquisition and retention of a spatial task in the Morris water maze and reduces cell genesis in the dentate gyrus (DG) of adult rats.[1] In the present study, the effects of nicotine and buproprion, an atypical antidepressant used in smoking cessation, on dentate gyrus cell genesis and DNA fragmentation were investigated. The results show that nicotine, chronically infused for 21 days, suppressed cell genesis and enhanced DNA fragmentation in the DG, an effect not attenuated by co-administration of buproprion.

## Introduction

The ability of the hippocampal formation, typically in its dentate gyrus (DG) area, to generate new neurons (neurogenesis) throughout the human lifespan[2] may prove beneficial in the treatment of neurological diseases characterised by neuronal cell loss such as Alzheimer's and Parkinson's diseases. Increased neurogenesis can be produced by a variety of treatments including an enriched environment[3], physical activity[4] and antidepressant drugs.[5,6] Neurogenesis has also been specifically implicated in learning tasks that involve the hippocampus.[7,8]

Clinical studies have revealed a strong correlation between the incidence of tobacco use and mood disorders.[9] In animal models, nicotine, infused chronically using a procedure similar to the one reported here, was found to have antidepressant properties.[10] Furthermore, nicotine dependence and withdrawal symptoms were ameliorated by buproprion[11,12], an atypical antidepressant approved for smoking cessation[13-15], probably via a mechanism involving the inhibition of nicotinic acetylcholine receptors (nAChRs).[16] *In vitro* studies revealed that buproprion exhibited some selectivity for neuronal nicotinic receptors that comprise $\alpha_3\beta_2$, $\alpha_4\beta_2$ and $\alpha_7$ subunits. Inhibition of radioactive nicotine binding to these receptor subtypes by buproprion suggested that the interaction was competitive and contributed to buproprion's efficacy in counteracting nicotine dependence.[17]

It is widely reported that nicotine produces a protective effect against induced apoptosis[18-21] in which one of the hallmarks is DNA fragmentation. Nevertheless, recent studies also suggest that nicotine enhances programmed cell death both in *in vitro* and *in vivo* systems[22-24] also at concentration levels such as those reported in smokers.[25,26] This study investigated the effects of constantly infused nicotine and buproprion on cell genesis (by determining BrDu incorporation) and DNA fragmentation in the DG. The nicotine dose chosen (4 mg/kg/day) results in blood nicotine concentrations (approximately 80 ng/ml) that would only be found in heavy smokers while the dose of buproprion (30 mg/kg/day) chosen was that reported to have antidepressant activity in the rat.[27] A 21-day chronic drug administration schedule was consistent with the time course for the therapeutic action of antidepressant treatment.

## Keywords

Nicotine, buproprion, bromodeoxyuridine, DNA fragmentation, hippocampus.

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## Materials and methods

### *Subjects*

Male Sprague-Dawley rats (Harlan Industries, UK), weighing 260-320g at the start of the experiment, were used. Rats were housed, two per cage, in a temperature-controlled (21°C) and humidity-controlled (50%±10%) environment on a 12 hour light/dark cycle with lights on at 6.00 am. Food and water were provided *ad libitum*. All the experiments were conducted during the light phase of the cycle and were in accordance with the UK Home Office regulations and covered by a Home Office project licence.

### *Drug treatment*

Rats were divided into four groups: a control group that received saline only (S-S), a nicotine-saline group that received 4 mg/kg/day nicotine and saline (N-S), a buproprion-saline group that received 30 mg/kg/day buproprion and saline (B-S), and a nicotine-buproprion group that received 4 mg/kg/day nicotine and 30 mg/kg/day (N-B) (Table 1). All reagents were purchased

from commercially available sources unless otherwise indicated. Nicotine hydrogen tartrate (Sigma, UK) and buproprion (gift from GlaxoSmithKline, UK) were dissolved in 0.9% saline solution. The dose of nicotine hydrogen tartrate was calculated as that of the free base. Before filling the osmotic minipumps, drug solutions and vehicle were sterilised by filtration through a 0.2μm filter.

Osmotic minipumps (Alzet®, ALZA Corporation, Palo Alto, CA, USA) were filled with drugs or vehicle as instructed by the manufacturer. They were implanted subcutaneously in the flank under inhalational anaesthesia (5% halothane for induction, 3% for maintenance) through a small incision on the back at the level of the shoulders on day 1 of the experiment. Two minipumps per rat, one on each side of the shoulder blade, were implanted (Table 1).

***Measurement of BrDu incorporation***

Cells formed from dividing progenitors were identified using bromodeoxyuridine (BrDu) (Sigma, UK) which integrates into DNA during the S-phase of DNA synthesis.[28] BrDu was dissolved in 0.9% saline and administered (50 mg/kg) on days 16-18 following implantation of minipumps. On day 21, rats were deeply anaesthetised with an overdose of pentobarbital sodium and perfused transcardially with 40 ml of saline followed by 140 ml of ice-cold paraformaldehyde (4% in 0.2 M sodium phosphate buffer, p.H. 7.4). Brains were removed and postfixed in paraformaldehyde solution for at least 24 h. Coronal sections (20 μm) were cut throughout the hippocampus using a cryostat. Every eighth section was thaw-mounted on slides.

Immunohistochemical techniques were used to identify the cells that had incorporated BrDu during cell division. A neuronal nuclear protein marker (NeuN) was used to help visualise the neurones within the hippocampus and identify the borders of the DG. To maximise antigen retrieval the following pretreatment steps were followed: DNA denaturation was performed by incubating the slides in 0.01 M citric acid (pH 6.0, 100°C, 10 min) followed by membrane permeabilisation (0.01% trypsin in 0.1 M Tris/0.02 M $CaCl_2$, 10 min) and acidification (2 M HCl, 25 °C, 30 min). Non-specific binding of primary antibodies was blocked by incubating the slides in the blocking buffer PBST [phosphate buffered saline (PBS) containing 0.25% Triton X-100 and 10% normal horse serum] for 1 h. The slides were then exposed to primary antibodies; mouse-anti-NeuN (Chemicon International, UK; 1:50) and rat-anti-BrDu (Harlan Sera-Lab, UK; 1:200) in PBST for 3 days at 4 °C. Sections were then washed in PBS for 10 min, blocked in PBST for 30 min and incubated with secondary antibodies [fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG, Scottish Antibody Production Unit (SAPU); 1:50 and tetramethylrhodamine isothiocyanate (TRITC)-labelled anti-rat IgG, SAPU; 1:160] for 1 h at room temperature. The slides were then washed in PBS for 10 min and after adding a few drops of Vectorshield (Vector Laboratories, UK) coverslips were placed on the slides and sealed with clear nail varnish.

Slides were coded before counting to ensure objectivity. BrDu-labelled cells were visualised using a fluorescent microscope (Zeiss Axioskop II). Alternate sections were selected for analysis. Hence, eight hippocampal sections per rat were taken at intervals of 320 μm. All BrDu-labelled cells within the DG were counted and the number of BrDu-labelled cells for each subject was expressed as a mean per section.[29]

***Analysis of DNA fragmentation***

DNA fragmentation was detected by the non-isotopic TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling] method using fluorescein-FragEL™ DNA fragmentation detection kit (Oncogene, UK) as instructed by the manufacturer. In brief, the sections were gently immersed in Tris buffered saline (TBS) (20 mM Tris pH 7.6, 140 mM NaCl) for 15 min at room temperature. They were then covered with 100 μl of 20 μg/ml proteinase K for 10 min followed by washing in TBS. The sections were then incubated at room temperature for 10 – 30 min in TdT equilibration buffer and 60 μl of TdT Labelling Reaction Mixture was then applied onto each section and covered with a parafilm coverslip. The slides were then incubated at 37°C for 1 – 1.5 h in a humidified chamber followed by washing in TBS. The sections were mounted with a glass coverslip using the provided mounting media, sealed with nail polish and visualised using a standard fluorescein filter. Two hippocampal sections per rat at interval of 1.5 mm were taken and fluorescein-labelled cells in both the right and left DG of each section were counted and the number of fluorescein-labelled cells for each subject was expressed as a mean per section.

***Data analysis***

All data was analysed using the Statistical Package for Social Scientists (SPSS, Version 11.5) and the level of statistical significance was taken as $p<0.05$. One-way analysis of variance (ANOVA) was used to determine group differences in BrDu and fluorescein-labelled cells (following data transformation for ranks) in the DG and post hoc using Tukey's HSD test.

## Results

***Effect of nicotine and buproprion on BrDu incorporation in the DG***

Chronic nicotine infusions reduced the number of BrDu-labelled cells within the the DG by approximately four-fold compared to rats infused with saline only (Figure 1). Statistical analysis revealed a significant chronic nicotine treatment effect [$F(1, 24) = 118.89$, $p<0.001$]. No significant treatment effect on the number of BrDu-labelled cells was observed following chronic infusion with buproprion [$F(1, 24) = 0.66$, NS] or between the two treatment effects [$F(1, 24) = 0.15$, NS] denoting that nicotine reduced the number of BrDu-labelled cells irrespective of the presence of buproprion. Post hoc analysis confirmed that only the administration of nicotine reduced the number of cells produced compared to saline ($p<0.001$).

***Effect of nicotine and buproprion on DNA fragmentation***

Compared to the saline group, groups infused with nicotine increased the number of fluorescein-labelled cells in the DG (figure 2). Statistical analysis revealed a significant chronic nicotine treatment effect [F (1, 24) = 18.43, $P$ <0.001] with no significant treatment effect on the number of fluoresceine-labelled cells following chronic infusion with buproprion [F (1, 24) = 0.21, NS] or between the two treatment effects [F (1, 24) = 0.27, NS] denoting that nicotine increased the number of fluorescein-labelled cells irrespective of the presence of buproprion. Post hoc analysis confirmed that only the groups in which nicotine was administered showed a significant increase in fluorescein-labelled cells and hence DNA fragmentation compared to saline (p<0.05).

## Discussion

This study has shown that constant infusion of nicotine, irrespective of buproprion co-administration, produced a significant reduction in BrDu incorporation and enhanced DNA fragmentation within the DG of the hippocampal formation. Also, the administration of buproprion alone did not produce any significant cellular changes compared to saline-treated rats. The latter is in contrast with other classes of antidepressant drugs such as the tricyclic antidepressants, monoamine oxidase inhibitors and selective serotonin reuptake inhibitors which has been shown to up-regulate neurogenesis in the DG.[30,31] Although the mechanism of action of buproprion is not yet fully understood, the drug is believed to inhibit dopamine and noradrenaline uptake more potently than serotonin uptake.[27,32] Such buproprion-induced inhibition of dopamine and noradrenaline transporter function and the resultant increase in extracellular dopamine and noradrenaline levels may substitute for nicotine-evoked neurotransmitter release during smoking, although nicotine reinforcement primarily has been associated with increased dopamine release.[33]

Together with nicotine replacement therapy, sustained release buproprion is efficacious as an aid in smoking cessation[32], possibly by acting as a nAChR antagonist.[17] However, this study showed that co-administration of buproprion did not influence nicotine-induced changes in cell genesis and DNA fragmentation with sustained administration denoting that the effects of nicotine probably do not depend upon stimulation of receptors antagonised by buproprion or by nicotine-evoked increase in dopamine or noradrenaline overflow. Also, the action of buproprion as a nAChR antagonist may be dose-dependent. In a self-administration paradigm in rats, Rauhut *et al.*[34] showed that treatment with buproprion decreased nicotine administration but only at the higher dose tested (78 mg/kg). In a similar experiment, Shoaib *et al.*[12], using the same dose of buproprion as in our experiment, injected daily for 28 days, failed to reduce nicotine-self administration in rats. Any nAChR antagonism at a higher buproprion dose is therefore subject to further experimental analysis.

Stressful stimuli have been reported to inhibit cell genesis in the hippocampus.[35] Nicotine, when administered at high doses, was found to have anxiogenic properties.[36,37] Thus, it is possible that the effects of the higher dose of nicotine on BrDu incorporation in the hippocampus could reflect the stress evoked by nicotine administration. However, the studies that demonstrated an anxiogenic response to nicotine employed subcutaneous injections of the drug. In the present investigation, nicotine was given by slow infusion from a subcutaneous minipump that avoided the high peak in nicotine evoked by the administration of a subcutaneous bolus of the drug. Additionally, previous studies have shown that nicotine infused at the dose presented here does not elicit an anxiogenic response, at least when it is investigated using the elevated plus maze test of anxiety.[38] These results imply that the chronic administration of nicotine by this route is not, in itself, stressful nor does it influence the response to an anxiogenic stimulus, such as the plus maze.

In this study, nicotine was also found to enhance DNA fragmentation, a hallmark of cell death via apoptosis or necrosis, in a dose that has previously been found to reduce spatial learning in the Morris water maze.[1] The mechanism by which nicotine induces cell death is still unclear. Berger *et al.*[25], and more recently Gimonet *et al.*[24], argue that $\alpha_7$ nAChRs are important

***Table 1:*** *Experimental protocol*
*Minipumps (M) containing nicotine, buproprion or the saline vehicle were inserted on day 1 of the experiment. On days 16-18, the animals were injected with BrDu as described in the* Materials and Methods *section and transcardially perfused on day 21 followed by brain sectioning for immunohistochemical processing.*

| ***Drug treatment*** | ***Day 1*** | ***Days 16-18*** | ***Day 21*** |
|---|---|---|---|
| Saline-Saline (S-S) | M1: saline;<br>M2: saline | BrDu | Transcardial perfusion |
| Saline-Nicotine (S-N) | M1: saline;<br>M2: nicotine (4 mg/kg/day) | BrDu | Transcardial perfusion |
| Saline-Buproprion (S-B) | M1: saline;<br>M2: buproprion (30 mg/kg/day) | BrDu | Transcardial perfusion |
| Nicotine-Buproprion (N-B) | M1: nicotine (4 mg/kg/day);<br>M2: buproprion (30 mg/kg/day) | BrDu | Transcardial perfusion |

***Figure 1:*** *The influence of chronic nicotine and buproprion infusions on the number of BrDu-labelled cells in the DG. Nicotine administration, in the presence of saline (S-N, n=6) or buproprion (N-B, n=6) reduced the number of BrDu-labelled cells compared to rats receiving saline only (S-S, n=6), an effect not reported following administration of buproprion alone (S-B, n=6). Results are shown as mean + SEM (*** p<0.001 compared to S-S by Tukey's HSD test). Inset above: Representative black and white photomicrographs in a section of the DG showing cells labelled for BrDu (Scale bar 50 μm).*

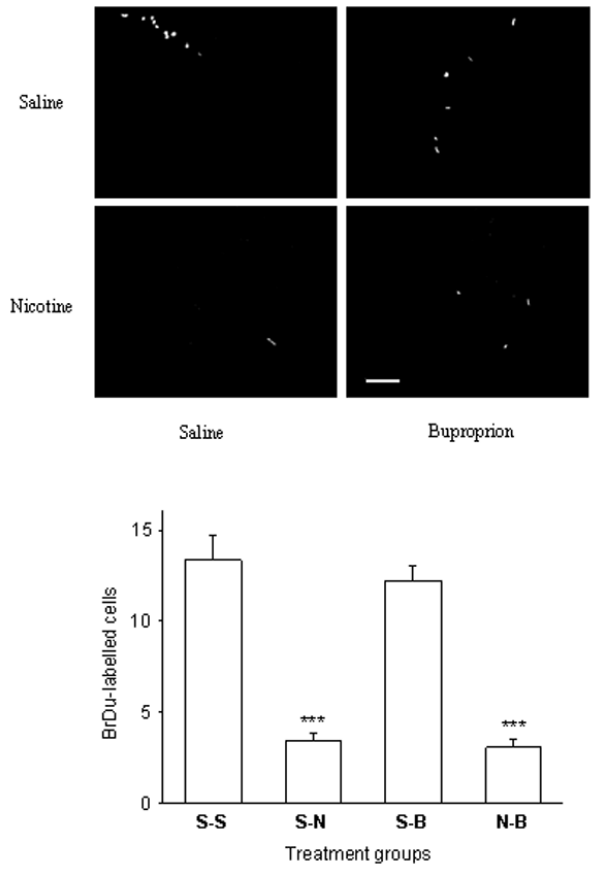
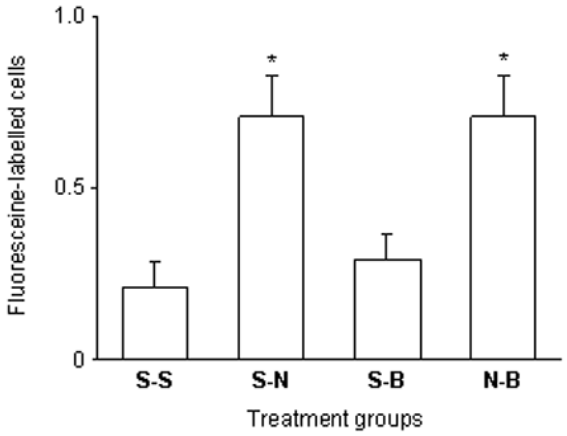



***Figure 2:*** *The influence of chronic nicotine and buproprion infusions on the number of fluorescein-labelled cells in the DG. Nicotine administration, in the presence of saline (S-N, n=6) or buproprion (N-B, n=6) enhanced DNA fragmentation compared to rats receiving saline only (S-S, n=6), an effect not reported following administration of buproprion alone (S-B, n=6). Results are shown as mean + SEM (*p<0.05 compared to S-S by Tukey's HSD test).*




in mediating these effects, possibly by playing an important role as modulators of synaptic strength in the CNS[39,40], and in processes involved in the pathophysiological changes observed in neurodegenerative diseases.[41,42] The importance of these receptors during neural development indicates that they are crucial in the early stages of development and differentiation.[43,44] Also, nicotine appears to have contradictory effects on cell survival in different systems showing both protective[45,46,47] and cytotoxic[23,24,26] properties. The effect of nicotine on cell survival probably depends on a number of factors such as specific gene expression, cell cycle stage, developmental stage, levels of trophic factors, and calcium-buffering capabilities.[48] The complex interaction of these effects could determine both the cytotoxic and protective effects of nicotine.

In conclusion, this study has shown that the constant infusion of nicotine reduces cell genesis and enhance cell death in the DG. These effects were not attenuated by co-administration of buproprion denoting the effects of nicotine probably do not depend upon stimulation of receptors antagonised by buproprion or by nicotine-evoked increase in dopamine or noradrenaline overflow. These observations could have important implications on the understanding of the role of nicotinic cholinergic systems in neurodegenerative disorders.

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