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Continuous administration of a selective $\alpha 7$ nicotinic partial agonist, DMXBA, improves sensory inhibition without causing tachyphylaxis or receptor upregulation in DBA/2 mice

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Abstract

Stimulation of nicotinic receptors, specifically the $\alpha 7$ subtype, improves sensory inhibition and cognitive function in receptor deficient humans and rodents. However, stimulation with a full agonist, such as nicotine, produces rapid tachyphylaxis of the P20N40-measured sensory inhibition process. 3-(2,4-dimethoxybenzylidene) anabaseine (DMXBA, also GTS-21) selectively activates the $\alpha 7$ nicotinic receptor, and in acute administration studies, has been shown to improve deficient sensory inhibition in both humans and rodents with repeated dosing. Unlike nicotine, this partial agonist acted without inducing tachyphylaxis. Here, we assessed the ability of DMXBA to improve sensory inhibition in DBA/2 mice after 7 days continuous administration via a subcutaneously implanted osmotic minipump. When assessed on day 8, mice receiving saline showed the characteristic deficient sensory inhibition seen with untreated DBA/2 mice. The 25 and 50 mg/ml infusion concentrations of DMXBA, but not the 100 mg/ml, produced significantly improved sensory inhibition in the mice, exclusively through a decrease in test amplitude. No concentration significantly upregulated hippocampal $\alpha 7$ receptor levels. DMXBA levels in the brain were higher than plasma at 2 of the 3 concentrations infused. These data suggest that continuous exposure to DMXBA does not significantly affect the underlying responsiveness of the sensory inhibition pathway to this partial agonist, nor cause receptor upregulation, at these relatively low brain concentrations. The ability of DMXBA to maintain its effectiveness during constant administration conditions may be due to an ability to activate $\alpha 7$ receptors at low concentrations, and consequently low fractional occupancy of the five possible binding sites on this homomeric receptor.

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Keywords

nicotinic receptors; schizophrenia; auditory gating; sensory inhibition; DMXBA; GTS-21

Introduction

Nicotinic receptors are members of a class of receptors (ligand-gated ion channels) that undergo rapid desensitization after agonist binding. This is particularly true for the $\alpha 7$ subtype (Quick and Lester 2002), which is present at relatively high concentrations in the hippocampus (see Nashmi and Lester 2006 for review). This rapid desensitization is perceived as a major problem that might limit the effectiveness of nicotinic agonists as drugs. An $\alpha 7$ receptor agonist $\alpha 7$ would be particularly useful for the treatment of sensory processing deficits occurring in certain mental disorders, including schizophrenia and bipolar depression (Leonard et al 2001). The sensory processing deficit is a failure of an inhibitory circuit in the hippocampus to suppress the response to repeated sensory stimuli, especially auditory stimuli. This failure to suppress the extraneous stimuli results in poor attention and, consequently, difficulties in learning and memory. It can also lead to sensory overload or “flooding,” which may lead to “personality decompensation” (Venables 1964; 1992). This is particularly relevant in schizophrenia.

Examination of postmortem brains of both normal individuals and schizophrenia patients has shown a reduction in the number of hippocampal $\alpha 7$ nicotinic receptors in the patients (Breese et al 1997). When administered a nicotinic agonist such as nicotine, or $\alpha 7$ selective agonists such as tropisetron or the partial agonist DMXBA, also known as GTS-21, schizophrenia patients show improvements in sensory processing (Adler et al 1993; Koike et al 2005; Olincy et al 2006) as well as in learning and memory (Harris et al 2004; Olincy et al 2006; Freedman et al, 2008).

Poor sensory processing (also termed auditory gating, or sensory inhibition) can be measured using a paired auditory stimulus paradigm (Olincy and Stevens 2007). Specifically, the sensory processing paradigm measures the ability of the person to inhibit the electrophysiological response to repeated stimuli. Paired identical auditory stimuli are presented at a short interstimulus interval (500 msec) and the response to each stimulus is measured and compared. In normal sensory processing, or inhibition, the response to the second, or test, stimulus is suppressed compared to the response to the first, or conditioning stimulus. In schizophrenia, the responses are of similar magnitude, indicating no inhibition of the response to the second stimulus (Freedman et al 1983; Adler et al 1998). The deficit can be modeled in DBA/2 mice which show poor sensory inhibition and also have reduced numbers of $\alpha 7$ receptors in the hippocampus (Stevens et al 1996). These mice also show improvement in sensory inhibition when administered nicotine, another full agonist, ABT-418 (Stevens and Wear 1997), or DMXBA (Stevens et al 1998) in an acute administration paradigm. However, if the agonist is administered a second time, at a relatively short interval (40 minutes), only DMXBA shows a repeated period of improved sensory inhibition. This is thought to be due to the partial agonist properties of DMXBA as compared to the full agonist effects of nicotine or ABT-418, which presumably induce a profound receptor desensitization. To assess whether DMXBA retains its ability to improve sensory inhibition when administered chronically at a relatively constant brain concentration, we measured its effectiveness after continuous administration to DBA/2 mice for 7 days.

Results

The parameters analyzed were: conditioning amplitude (amplitude of the response to the first stimulus), test amplitude (amplitude of the response to the second stimulus) and TC ratio (test amplitude/conditioning amplitude) which gives the measure of inhibition. When TC ratios for all doses of DMXBA and vehicle control were analyzed by ANOVA, significant differences were observed ($F(3,45)=20.03$, $p<0.001$). A *posteriori* analysis showed the 25 and 50 mg/ml doses had significantly reduced TC ratios compared to vehicle control, but were not different from each other (Figures 1 and 2). Analysis of conditioning and test amplitudes revealed significant reduction in test amplitude ($F(3,45)=15.08$, $p<0.001$), while there was no difference in conditioning amplitude across doses (Figure 1). A *posteriori* analysis of the test amplitudes showed that all 3 doses were significantly reduced as compared to control, but not different from each other.

ANOVA for the level of [125 I]- α -bungarotoxin binding in the hippocampus across the doses of DMXBA did not show significant changes in binding in any region (Figure 3), although examination of the figure suggests that there may be a small dose-dependent increase in binding with the higher doses (50 and 100 mg/ml) that may not have reached significance due to issues of group size.

Analysis of brain and plasma levels for DMXBA after 7 days of continuous administration showed the expected significant difference in brain levels ($F(3,12)=4.028$, $p=0.034$), however, not in plasma levels (Figure 4). The data show successive increases in brain level as the concentration of DMXBA in the pump increases, with the 100 mg/ml infusion leading to a brain concentration significantly different from the vehicle. Interestingly, the levels in plasma only approached significance ($F(3,12)=2.995$, $p=0.077$) (Figure 4). When brain and plasma levels were compared at each concentration, only the 100 mg/ml infusion showed a significant difference between plasma and brain concentrations.

Discussion

Studies have established the role of hippocampal $\alpha 7$ nicotinic receptors in normal sensory inhibition as demonstrated with the paired-auditory-stimulus paradigm. Rodents with reduced levels of these receptors show reduced inhibition (Stevens et al 1996; Adams et al 2008). Intraventricular administration of an $\alpha 7$ antagonist (α -bungarotoxin) in rats with normal $\alpha 7$ receptor levels, induces abnormal sensory inhibition (Luntz-Leybman et al 1992). Postmortem brains of human schizophrenia patients have reduced numbers of hippocampal $\alpha 7$ nicotinic receptors as compared with brains of normal individuals (Freedman et al 1995) and these patients show deficient sensory inhibition (Adler et al 1998). In individuals with reduced hippocampal $\alpha 7$ nicotinic receptors, be they rodent or human, stimulation of the remaining receptors improves sensory inhibition. This occurs with direct (Adler et al 1993, Stevens and Wear 1997) or indirect stimulation (Nagamoto et al 1996; 1999; Simosky et al 2003; 2009).

DMXBA selectively stimulates (as a partial agonist) the $\alpha 7$ nicotinic receptor but also, at higher (micromolar) concentrations, acts as an antagonist at $\alpha 4\beta 2$ receptors (Briggs et al 1995; de Fiebre et al 1995; Kem et al 2004; Stokes et al 2004). Studies have demonstrated improvement in sensory inhibition with DMXBA administration in both schizophrenia patients (Olinco et al 2006) and DBA/2 mice (Stevens et al 1998). In addition, improvement in some aspects of cognition, including attention, in healthy young adults (Kitagawa et al. 2003), schizophrenia patients (Freedman et al 2008) and in a variety of animal species and disease models (Kem 2000; Buccafusco et al. 2005) have been observed. Desensitization of the $\alpha 7$ receptor displays concentration-as well as time-dependence; it quickly desensitizes at

agonist concentrations that allow occupation of most of the five possible binding sites present in each receptor (Papke et al. 2000; Quick and Lester 2002). Thus, the potential exists for agonist-induced desensitization with repeated or chronic administration of agonists at concentrations sufficient to occupy most of these sites. Tests with repeated acute administration of DMXBA in DBA/2 mice demonstrated that 2 identical injections, 40 minutes apart did not produce desensitization as shown by repeated improvement in sensory inhibition after both injections (Stevens et al 1998). The present study sought to determine if continuous systemic administration of DMXBA to DBA/2 mice would induce receptor desensitization or if improvement in sensory inhibition would still be produced. The data show that, even after 7 days of continuous administration, mice receiving the 25 and 50 mg/ml DMXBA infusions had improved sensory inhibition. Infusion of the highest concentration (100 mg/ml) of DMXBA failed to show an improvement in sensory inhibition as defined by the TC ratio, although it appears that it should by comparison of the conditioning and test amplitudes. This is because each parameter (conditioning amplitude, test amplitude and TC ratio) is an average of all animals' data for that parameter. The TC ratio is therefore the average of all the TC ratios, not the ratio of the mean test amplitude/the mean of the conditioning amplitude. So in this particular case the mean TC ratio is higher than would be presumed from the appearance of the mean conditioning or test amplitude. None-the-less, a reduction in test amplitude specifically demonstrates inhibition so that, even though the mean TC ratio for this concentration is not significantly lower than baseline, inhibition of the second response has taken place. The simplest explanation for the lack of significant change in TC ratio is that, at this high brain DMXBA concentration (Fig. 4) many of the receptor sites were occupied by DMXBA, such that a large fraction of the receptors were in a desensitized state. Nicotinic agonists are known to display reduced effects at high concentrations (*in vitro*) and doses (*in vivo*). Suggestion of a so-called inverted U-shape dose dependence of DMXBA action was also observed in clinical trials (Olinicy et al. 2006; Freedman et al. 2008; Tregellas et al. 2009).

The improvement in sensory inhibition we observed with continuous administration of DMXBA was very similar to what we previously observed with acute administration. That is, the improvement was produced solely through a significant decrease in test amplitude, signifying $\alpha 7$ nicotinic receptor activation (Stevens et al 1998). A lack of change in conditioning amplitude was expected, since $\alpha 7$ receptor agonists in general do not affect conditioning amplitude and, due to DMXBA antagonism at the $\alpha 4\beta 2$ nicotinic receptor, if it occurred in our experiments, would not be expected to affect this response (Radek et al 2006; Wildeboer and Stevens 2008). In the latter study, administration of the relatively selective $\alpha 4\beta 2$ -receptor antagonist dihydro- β -erythroidine, did not alter conditioning amplitude. Therefore, in the present study, the possible blockade of $\alpha 4\beta 2$ receptors by DMXBA would not be expected to affect conditioning amplitude. Altogether, these data suggest that tonic control of the conditioning amplitude is not through nicotinic receptors, but via another neurotransmitter-receptor system.

Prolonged exposure to nicotine can lead to upregulation of $\alpha 4\beta 2$ and $\alpha 7$ nicotinic receptors, but upregulation of the latter subtype is smaller and only occurs at concentrations higher than are reached in smokers (Buisson and Bertrand 2002). Although previous studies of long term administration of DMXBA by daily injections in rats failed to demonstrate any upregulation of $\alpha 7$ receptors (4 weeks, Bjugstad et al. 1996; two weeks, Meyer et al 1997), we anticipated that continuous delivery of DMXBA might upregulate $\alpha 7$ receptors. A statistically significant effect on receptor concentration was not observed in the present study. However, examination of the binding data (Fig. 3) suggests that in all hippocampal regions assessed, infusions at the two highest concentrations may have slightly increased binding.

There was a tendency for the DMXBA to be elevated in the brain tissue relative to the plasma. This is similar to the pattern observed following acute subcutaneous injection (Stevens et al 1998), but not after oral administration (Simosky et al 2001) of DMXBA. In rats administered larger doses of DMXBA orally by gavage or by intraperitoneal injection, the total brain levels are elevated with respect to plasma levels (Mahnir et al 1998; Kem et al 2004). It is possible that the lack of elevated levels in the brain, relative to plasma in the mouse oral administration study was due to a lower dose being given, as first-pass hepatic metabolism of DMXBA is quite large in rats and man (>70%). It must be noted that our brain and plasma estimates of DMXBA pertain to its total concentration, not its free concentration. The free concentrations are predicted to be much smaller (Kem et al 2004).

In summary, continuous delivery of DMXBA, at 25 and 50 mg/ml, 1.0 μ /hr (25 and 50 μ g/hr, respectively), improved sensory inhibition in DBA/2 mice, while apparently not producing response tachyphylaxis. One possible explanation for this lack of decrement in response (its magnitude here was very similar to our original acute study effect; see Stevens et al. 1998) is that DMXBA somehow protects at least some receptors from desensitization, so they remain available for continuous stimulation by this partial agonist. It was recently demonstrated in a crystallographic analysis of DMXBA binding to the acetylcholine binding protein, a protein model for the five ACh-binding sites of homomeric nicotinic receptors like the $\alpha 7$ receptor, that DMXBA binds in two different conformations, one being agonist-like and the other being antagonist-like, within the same pentameric complex (Hibbs et al 2009). If this also takes place within the $\alpha 7$ receptor, it might reduce the probability of complete desensitization as well as the probability of full activation. Another important property of $\alpha 7$ agonists is that their cognitive effects *in vivo* occur at concentrations significantly below what would be predicted from studies of acute electrophysiological responses in cells expressing the receptor which are suddenly exposed to the agonist (Papke and Porter-Papke 2002; Bitner et al 2007; Leiser et al 2009). This was probably due to the ability of the $\alpha 7$ receptor to remain activated (open channel) at very low agonist concentrations that favor occupation of one or two of the five possible binding sites present on each receptor (Papke et al 2000).

The sensory inhibition improvement by DMXBA was produced, as expected, by a significant reduction in test amplitude. DMXBA did not produce significant upregulation of the $\alpha 7$ receptors. The lack of inhibitory-response tachyphylaxis during continuous exposure suggests that DMXBA and similar partial agonists could be promising drug candidates for potential human therapeutic use. A daily dosing clinical trial of DMXBA has been completed (Freedman et al 2008). Sensory inhibition was not assessed in this study, but patients on DMXBA showed significant improvement in certain tasks measuring attention, a cognitive function thought to be modulated in part by $\alpha 7$ nicotinic receptors (Leiser et al 2009; Roncarati et al 2009).

Experimental Procedure

Male DBA/2 mice weighing 20–25 gm were briefly anesthetized with isoflurane and implanted in the upper back with a subcutaneous osmotic minipump (Alzet, Durect Corp. Cupertino, CA) which delivered 1 μ /hr for 7 days. The pumps were loaded with filter-sterilized (0.22 μ M) DMXBA (25, 50 or 100 mg/ml) in physiological saline or saline alone. On the 8th day after pump implantation, the mice were recorded for sensory inhibition. The mice were anesthetized with chloral hydrate (400 mg/kg, ip) and pyrazole (400 mg/kg, ip) to retard the metabolism of the chloral hydrate. Mice were placed in the stereotaxic instrument and the scalp incised. A burr hole was opened over the dorsal hippocampus and another over the contralateral anterior cortex. A stainless-steel, tefloncoated recording electrode (127 μ M diameter) was lowered to the CA3 region of the dorsal hippocampus, [1.8 mm posterior to

bregma, 2.7 mm lateral to midline ~1.6–1.8 mm ventral to dura (Paxinos and Franklin 2003)]. Final placement was determined by the presence of complex spike activity typical of hippocampal pyramidal neurons (Miller and Freedman 1995). An identical electrode was placed on dura over the anterior cortex to act as a reference. Miniature earphones attached to hollow ear bars, placed at the externalization of the aural canal, delivered the auditory stimuli. EEG responses to paired click stimuli (3000 Hz, 10 ms, 72 dB SPL, presented 0.5 sec apart, with 9 sec between pairs) were amplified 1000 times with bandpass filtering at 1–500 Hz. Data were collected, stored and analyzed using SciWorks (DataWave, Berthoud, CO). The responses to 16 pairs of stimuli were averaged to constitute a record and 12 records were obtained at 5-minute intervals for each animal to characterize its sensory inhibition parameters. Sensory inhibition parameter data were averaged across the 12 records and analyzed by one-way ANOVA with Tukey's HSD, or Tukey-Kramer, *a posteriori* analyses where appropriate.

The complex of interest in the sensory inhibition paradigm occurs between 20 and 60 msec after stimulus onset. The amplitude of the wave was calculated from the peak of the positivity to the peak of the negativity. This complex has been shown to have less variability than either component alone (Hashimoto et al 2005). Three parameters were assessed: conditioning amplitude—the amplitude of the response to the first, or conditioning, stimulus; test amplitude—the amplitude of the response to the second, or test, stimulus; and TC ratio—test amplitude/conditioning amplitude. The TC ratio is the measure of inhibition; ratios <0.05 are considered to be normal sensory inhibition (Freedman et al 1996).

To assess regional levels of hippocampal $\alpha 7$ nicotinic receptor binding, some mice were sacrificed by decapitation at the end of the recording session without regaining consciousness. The brain was removed and frozen in dry ice snow. Transverse sections (20 μ M) through the dorsal hippocampus were collected for generation of [125 I]- α -bungarotoxin autoradiograms. The tissue sections were incubated in a solution containing 50 mM Tris-HCl, 120 mM NaCl and 2 mg/ml bovine serum albumin (Tris/BSA buffer, pH 7.4) either with (nonspecific condition) or without (total condition) 5 mM nicotine for 30 minutes at room temperature. The tissue sets were then be incubated in Tris/BSA buffer containing [125 I]- α -bungarotoxin (5 nM, specific activity 2000 Ci/mmol, Perkin Elmer, Waltham, MA) at 37° C for 3 hours. At the conclusion of the incubation period, the tissue was rinsed in Tris/BSA buffer for 5 minutes, in Tris buffer without BSA for 15 minutes, and in phosphate buffered saline (pH 7.4) for 5 minutes, all at 37°C. The tissue sections were briefly dipped in distilled water, dried in a stream of cool air, and apposed to β -max film, with [14 C] standards of known radioactivity, at room temperature for 48 hours (GE Healthcare-Biosciences Corp., Piscataway, NJ). Autoradiograms were then quantified with a computer-based image analysis system (C-Imaging Systems, Compix, Inc., Cranberry Township, PA) using calibrated standards of reference. A calibration curve of optical density against radioligand concentration (fmol/mg tissue) was constructed using the standards. Grey values in discrete regions of the autoradiographic images were measured and corresponding values of radioactivity determined by interpolation from the calibration curve. Fifteen sections per mouse were analyzed and average binding determined for the dentate gyrus, CA1 and CA3 regions of the hippocampus for each mouse. Averaged binding densities for the 3 regions of the hippocampus were compared across the three doses by one-way ANOVA.

For the UPLC-MS/MS determinations of DMXBA concentrations in brain and plasma samples, DMXBA stock solution A (1 mg/mL in DMSO) was prepared, then a 0.4 mg/mL working solution was prepared from stock A by diluting 200 μ L into 300 μ L of DMSO. A 200 ng/ml reserpine internal standard working solution was prepared in acetonitrile. The standard curve was prepared by aliquoting 50 μ L of control mouse plasma or blank mouse brain solution (3:1 w/v with water) into 12 wells of a 96-deep well plate. The plasma or

brain solution was spiked with 2.5 μ L of the 0.4 mg/ml DMXBA working solution using a low volume multi-channel pipette to give a curve range from 0.1 to 20,000 ng/ml (the working range of the curve was from 3.0 to 2222 ng/ml for plasma and brain and from 9.0 to 20000 ng/ml for the tube samples). For the samples 50 μ L of mouse plasma or brains (prepared by homogenizing mouse brain samples 3:1 w/v with water) were aliquoted into a 96-deepwell plate and 2.5 μ L of blank DMSO was added. A 300 μ L aliquot of working internal standard solution was added to each sample and standard to precipitate the proteins. The tube wash samples were prepared by diluting the samples by 2000 and aliquoting 2 μ L into 350 μ L of the working internal standard solution. The standards and samples were vortexed for 5 minutes and centrifuged for 10 minutes at 13,000 g. The top layer was transferred to an injection vial and 10 μ L was injected into the Waters Acquity UPLC-MS/MS system.

A Waters Acquity UPLC-MS/MS system was used to analyze the samples. The mobile phase was 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The gradient was from 5–95% solvent B over 4.5 minutes with a flow rate of 600 μ L/min. A Water Acquity UPLC BEH-C18 1.7 μ M 2.1 $\times$ 50mm column was used for the separation.

The mass spectrometric transitions were 309.2 to 171.2 m/z for DMXBA and 609.3 to 397.4 m/z for reserpine. The mass spectrometric parameters were collision voltage of 23 and 28 for DMXBA and reserpine, respectively with a cone voltage of 40. DMXBA brain and plasma levels were compared by *t*-test at each dose.

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Figure 1.

Mean conditioning and test amplitudes, and TC ratio for DBA/2 mice after 7 days of continuous subcutaneous administration of saline or DMXBA (25, 50 or 100 mg/ml, 1 µl/hr). There were no significant changes in conditioning amplitude at any dose tested, however, all 3 doses of DMXBA produced significantly reduced test amplitudes compared to saline. TC ratios were significantly reduced for the 25 and 50 mg/ml concentrations. Data are mean $\pm$ SEM, $n=12$ per group, $^{***}p<0.01$, Tukey's HSD.

Figure 2. Representative wave forms for a mouse with saline in the osmotic pump and one with 50 mg/ml DMXBA in the pump. The mouse receiving DMXBA shows a reduced test amplitude while the mouse on saline does not. Stimulus initiated at the arrow, tick marks note the wave of interest.

Figure 3.

Mean levels of [125 I]- α -bungarotoxin binding, defining $\alpha 7$ nicotinic receptors, in regions CA1, CA3 and the dentate gyrus of the hippocampus of DBA/2 mice after 7 days continuous subcutaneous administration of saline or DMXBA (25, 50 or 100 mg/ml, 1 μ l/hr). There were no significant differences in binding in any region at any dose tested. Data are mean $\pm$ SEM, $n=4$ per group

Figure 4.

Mean plasma and brain levels of DMXBA after 7 days continuous subcutaneous administration. At 100 mg/ml concentration of DMXBA in the pump, the brain level was significantly different from the saline control and significantly different from the plasma level at the same dose. A DMXBA (MW 381) total concentration of 100 ng/ml equals 0.26 μ M. In plasma samples the bound fraction is approximately 95% (Kem et al. 2004), so the free concentration in plasma and presumably brain will be much lower. Data are mean $\pm$ SEM, $n=4$ per group. * $p<0.05$ compared to saline, # $p<0.05$ compared to plasma.