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ORIGINAL INVESTIGATION

**Philip M. Wall · Claude Messier**

# Infralimbic kappa opioid and muscarinic M1 receptor interactions in the concurrent modulation of anxiety and memory

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**Abstract** *Rationale*: Spontaneous working memory and anxiety-like behaviour can be concurrently influenced following kappa opioid or muscarinic M1 antagonist infusions in the infralimbic (IL) area of the ventromedial prefrontal cortex (vmPFC) in CD-1 mice. Further dose–response analyses of our previous norBNI and pirenzepine data revealed significant dose × drug interactions on trial-1 and -2 anxiety-related elevated plus-maze indices. These data prompted us to evaluate the effects of simultaneous IL norBNI/pirenzepine infusions on anxiety and spontaneous working memory. *Objective*: The present study sought to evaluate whether (a) our previously reported anxiogenic and working memory disruptive effects of norBNI, and anxiolytic and working memory disruptive effects of pirenzepine data could be replicated using the most effective dose (10 nmol) of each drug and (b) IL infusions of mixed kappa/M1 receptor inhibitor drugs might interactively influence these cognitive, behavioural processes. *Methods*: Anxiety was evaluated in the elevated plus maze, and spontaneous alternation memory was evaluated in the Y-maze following pirenzepine, norBNI or two levels of norBNI/pirenzepine drug mix infusions in the IL vmPFC. *Results*: Pretreatment with the M1 antagonist pirenzepine was anxiolytic in trial 1 (10 nmol) and trial 2 (no-injection) in the elevated plus maze 24 h later, and disrupted alternation performance and some aspects of attention in the Y-maze. Pretreatment with the kappa antagonist norBNI was anxiogenic in trial 1 (10 nmol) and trial 2 (no-injection) in the elevated plus maze 24 h later, and also disrupted alternation performance and some aspects of attention in the Y-maze. The norBNI-10 nmol/pirenzepine-10 nmol mixed drug infusion was somewhat anxiogenic in trial 1, exerted no carry-over effects in trial 2 in the elevated plus maze, and disrupted alternation memory and some aspects of attention in the Y-maze. The norBNI-5 nmol/pirenzepine-10 nmol drug mix had no effect on trial-1 or -2 anxiety measures in the elevated plus maze, yet also disrupted Y-maze spontaneous memory performance. *Conclusions*: (1) The effects of IL infusions of norBNI or pirenzepine (10 nmol/0.5 µl) alone on anxiety-like behaviour and aversive learning and memory in the elevated plus-maze replicated previously reported data. (2) Mixed M1/kappa receptor inhibition in the IL cortex exerted counteractive effects on anxiety-like behaviour and aversive learning in the elevated plus maze. (3) Mixed M1/kappa receptor inhibition appeared to exert additive disruptive effects on alternation performance and aspects of attention related to active working memory in the Y-maze.

**Keywords** Concurrent modulation · Anxiety · Spontaneous working memory · Attentional mechanisms · Infralimbic vmPFC · Muscarinic1 and kappa receptors · Elevated plus-maze · Y-maze

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## Introduction

There are growing data demonstrating that the ventromedial prefrontal cortex (vmPFC) influences different forms of working memory processing in mice and rats (Poucet 1997; Fritts et al. 1998; Jung et al. 1998; Ragozzino and Kesner 1998; Wall and Messier 2000a, 2000c; Wall et al. 2001). The ventral prelimbic (PL) and infralimbic (IL) cortices have also been shown to be involved in anxiety regulation in mice and rats (Morgan et al. 1993; Morgan and LeDoux 1995; Duncan et al. 1996; Goldstein et al. 1996; Petty et al. 1997; Broersen et al. 2000; Wall and Messier 2000a, 2000c; Wall et al. 2001). Moreover, there are growing data showing that the vmPFC can concurrently regulate aversive learning, memory and anxiety-like behaviour in mice.

In our previous pharmacological studies, we examined the possibility that learning, memory and anxiety could be simultaneously influenced following drug infusions in the IL area of the vmPFC in CD-1 mice, using a two-trial strategy in the elevated plus maze (Wall and Messier 2000a, 2000c; Wall et al. 2001). It should be noted that there have been two different two-trial elevated plus-maze procedures used in pharmacological studies (File et al. 2000; Wall and Messier 2000c). In the procedure of File and colleagues, drug-naive animals were given trial 1 with no injection, followed by a pretrial-2 intracerebral drug injection 24 h later. In our studies, animals were given a pretrial-1 intracerebral drug injection, followed by a no-injection trial 2, 24 h later. The rationale behind the use of either of the two-trial procedural strategies depends on whether the experimenter wishes to (a) investigate drug effects on aversive learning – with subsequent long-term effects on memory 24 h later or (b) investigate drug effects on aversive memory in trial 2, 24 h after aversive learning presumably occurred during a no-drug trial 1.

Using a two-trial procedure in our confirmatory factor analysis (CFA) study primarily provided an opportunity to not only measure the degree of aversive learning that presumably occurs during trial 1 in drug-naive mice in the elevated plus maze (File 1993; File et al. 1993, 1998, 2000; Fernandes and File 1996; Espejo 1997; Holmes and Rodgers 1998; Ouagazzal et al. 1999; Kenny et al. 2000), but also to estimate the memory for such learning in trial 2, 24 h later (Wall and Messier 2000b). The large number of subjects used in our CFA study ($n$=200) also provided useful estimates of baseline behavioural indices for both trials. Indeed, the data from the measured anxiety indices (open arm entries, open arm time ratio, unprotected stretch attends, unprotected head dips) were reduced by about 50% in trial 2. Moreover, the trial-2 anxiety-related CFA data closely resembled the trial-1 anxiogenic data from our norBNI (Wall and Messier 2000a) and McN-A-343 (Wall et al. 2001) studies.

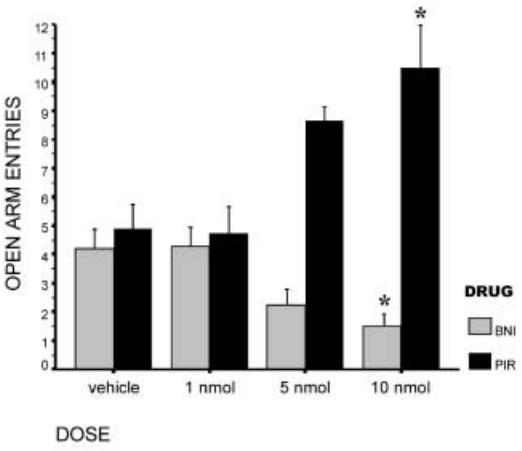
The fact that some type of associative 'aversive' learning during trial 1 potentiated aversive memory and subsequent anxiogenic responses in animals re-exposed to the elevated plus maze 24 h later indicated close relationships between associative learning and memory, and anxiety. Moreover, the neural mechanisms underlying these psychological relationships appear to be closely related. Consequently, we consistently use the pretrial-1 drug infusion two-trial testing strategy, primarily because we can evaluate drug effects on trial-1 aversive learning and trial-2 memory, and thus further investigate relationships between learning, memory and anxiety in the elevated plus maze.

As a further evaluation of IL drug infusions on memory, we also evaluated the effects of the same IL drug infusions on 'active' working memory [spontaneous alternation performance (SAP)] in the Y-maze. Because there has been some suggestion that the SAP ratio is related to active working memory, it can be considered a reasonable index of at least some aspects of working memory (Maurice et al. 1994; Kameyama et al. 1998; Ukai et al. 1998). However, several authors have suggested that so-called 'delay-independent' disruptive effects on working memory result from attentional disturbances (rather than memory deficits; Delatour and Gisquet-Verrier 2000; Robinson et al. 2000). It is therefore conceivable that since active working memory continuously operates in an awake behaving animal, then delay-independent disruptions (observed in laboratory testing situations) likely reflect effects on different aspects of active working memory, such as attention and working behavioural set (Fuster 1997; Wall and Messier 2001).

Our concept of "spontaneous 'active' working memory" is derived from the aforementioned notion that oftentimes working memory can be 'delay-independently' disrupted in animals. For example, SAP in mice in a T-maze conceivably stems from some sort of 'automatic' mnemonic processing (Tako et al. 1988). Moreover, it could be suggested that non-matching-to-sample tasks may tap into 'automatic' working memory processes (Granon et al. 1995). In both cases, animals can perform such tasks by utilising relatively 'automatic' (continuously active) memory processing strategies (Granon et al. 1995; Tako et al. 1988). Spontaneous foraging in an eight-arm radial maze has also been suggested to be an active form of working memory (Floresco et al. 1997; Seamans et al. 1998). These investigators suggested that while, in a delay-dependent working memory test in an eight-arm radial maze, rats engage in prospective working memory processing after a delay, in a spontaneous foraging situation, rats engage in retrospective working memory processing (Floresco et al. 1997). Conceivably, spontaneous foraging, non-matching-to-sample and SAP are driven by the same (or similar) active working memory processes (Wall and Messier 2001).

The behavioural data from our muscarinic receptor study clearly demonstrated that muscarinic M1 receptors in the IL cortex concurrently influenced anxiety, aversive learning and active working memory in CD-1 mice (Wall et al. 2001). Moreover there appeared to be a consistent relationship between aversive learning and memory, and anxiety in the elevated plus maze in our muscarinic and kappa receptor studies. Accordingly, anxiolytic or anxiogenic drug effects were coupled with disrupted or enhanced, respectively, aversive learning in trial 1 and memory in trial 2. We interpret these data to mean that there were consistent concurrent carryover drug effects on anxiety and memory in the elevated plus maze.

**Fig. 1** Effects of pretrial-1 infralimbic drug infusion interactions on anxiety-related behaviours in the elevated plus maze. Values represent mean±SEM. *Grey bars* represent norBNI, *black bars* pirenzepine. *Significantly different than respective controls ($P$<0.05). Trials 1 and 2 are shown separately for open arm entries (OE), open time ratio (OTR), unprotected head dips (UHD) and unprotected stretch attends (USA). The norBNI data were taken from Wall and Messier (2000a) and the pirenzepine data from Wall et al. (2001), with permission. Data sets were analysed together in two-factor analyses of variance to test for dose × drug interaction effects. There were significant interaction effects on each of the four measures for both trials ▶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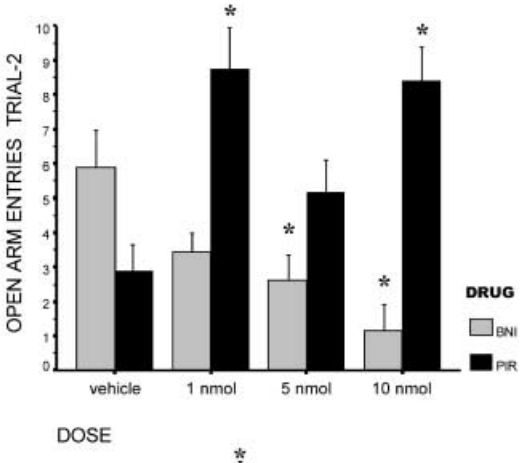
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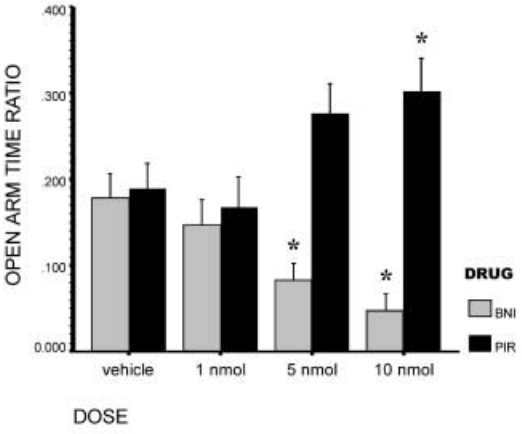
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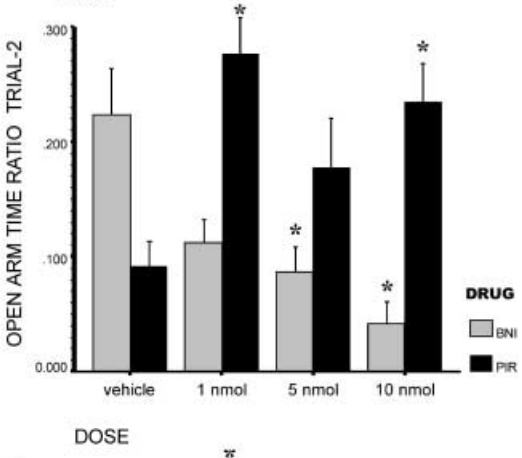
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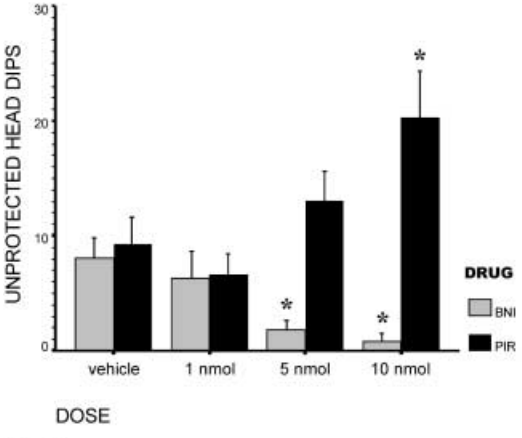
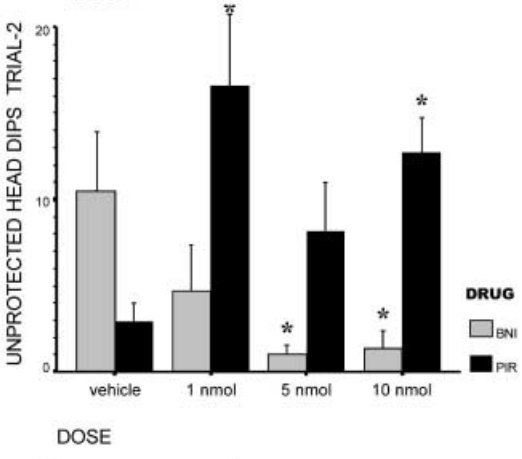

UNPROTECTED HEAD DIPS
vehicle
1 nmol
5 nmol
10 nmol
DOSE
DRUG
BNI
PIR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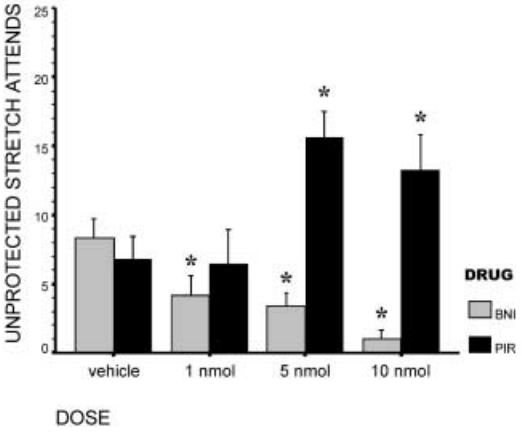
10 nmol
DOSE
DRUG
BNI
PIR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BNI
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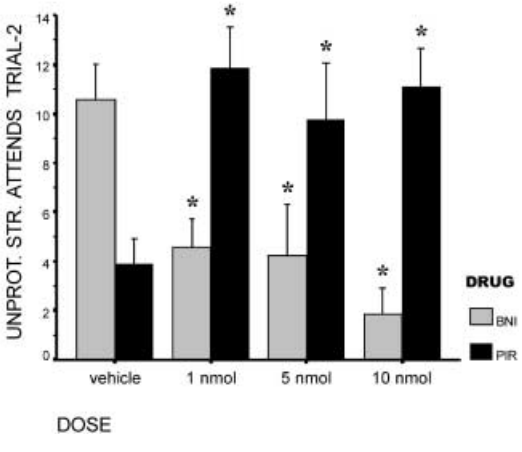
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There were some intriguing differences, however, between the effects of kappa drugs and M1 drugs on the relationship between anxiety and active working memory. While the kappa antagonist was anxiogenic and disrupted active working memory (Wall and Messier 2000a), the M1 antagonist was anxiolytic and also disrupted active working memory (Wall et al. 2001). In addition, while the kappa agonist was anxiolytic and enhanced active working memory (Wall and Messier 2000c), the M1 agonist was anxiogenic and enhanced active working memory (Wall et al. 2001). We realised that no simple relational 'rules' between anxiety and active working memory could be readily conceptualised. We did, however, hypothesize that since kappa and M1 receptor blockade produced opposite effects on anxiety and aversive learning and memory, and similarly disrupted active working memory, then both receptor mechanisms may interact in the vmPFC in the concurrent regulation of anxiety and memory in the elevated plus maze, and perhaps additively influence SAP and some aspects of attention related to active working memory in the Y-maze.

To test this possibility, we first took the dose–response data from our norBNI study (0, 1, 5 and 10 nmol doses; Wall and Messier 2000a) and corresponding dose–response data from our pirenzepine study (0, 1.25, 5, and 10-nmol doses; Wall et al. 2001), and ran two-factor analyses of variance (ANOVAs; drug × dose). Results for the anxiety measures (open entries, open time ratio, unprotected head dips, unprotected stretch attends) are depicted in Fig. 1. In all cases, there were significant drug × dose interaction effects in trials 1 and 2. These data provided evidence to support the hypothesis that simultaneous IL norBNI/pirenzepine infusions should exert concurrent counteractive effects on aversive learning, memory and anxiety in the elevated plus maze.

Given the growing data in support of the notion that cholinergic terminals in the mPFC mediate aspects of attentional processing that may determine the active formation of memory and on-going retrieval of information (Sarter and Bruno 1999), it is possible that the previously observed spontaneous alternation memory deficits following pirenzepine and norBNI infusions in the IL cortex may have also encompassed attentional disruptions as well. We reasoned that spontaneous exploration activity places attentional monitoring demands on cholinergic transmission in the vmPFC. This attentional monitoring activity might be crucial to the on-going operation of unfolding behavioural set activity (Gray and McNaughton 2000; Wall and Messier 2001). Conceivably, IL pirenzepine or norBNI infusions disrupted some aspects of attention in the Y-maze that are related to active working memory. It is therefore possible that simultaneous norBNI-pirenzepine drug infusions in the IL cortex may exert additive disruptive effects on some aspects of attention related to active working memory, compared with the disruptive effects of norBNI or pirenzepine alone.

In the present study, we primarily sought to evaluate whether concomitant kappa opioid and M1 receptor blockade in the IL cortex would exert counteractive effects on anxiety and aversive learning and memory in a 2-trial elevated plus-maze procedure, as well as exert additive effects on some aspects of attention related to active working memory in the Y-maze, in CD-1 mice.

## Materials and methods

### Subjects

Subjects were 60 male CD-1 naive mice, obtained from Charles River, St. Constant, Quebec. All mice were initially housed in polypropylene cages (groups of ten per cage) in a temperature-controlled room (21±1°C), with ad lib access to food and water. The mice were acclimatised for 6–8 weeks until they reached a weight range of 32–45 g. All mice were maintained on a 12-h/12-h light/dark cycle (lights on at 0700 hours).

All principles of laboratory animal care as specified in the NIH publication no. 85-23 (revised 1985), as well as specified by the animal care committee of the University of Ottawa and affiliated medical research facilities, were strictly followed.

### Surgery

Surgery was performed in accordance with the procedures of our laboratory elaborated elsewhere (Messier et al. 1999). Briefly, preoperative analgesia was provided by adding an acetaminophen solution (80 mg/5 ml; children's Tempra, Mead Johnson) to the drinking water bottles (1 ml Tempra in 100 ml $H_2O$) for 3 days prior to surgery. Fifteen minutes before surgery, mice were administered 0.01 mg/kg s.c. glycopyrrolate (Robinol, 0.01 mg/mouse; Wyeth-Ayerst, Montreal, Canada) to prevent accumulation of salivatory and bronchial secretions. Mice were then pre-medicated with sodium pentobarbitol (0.65 mg/ml) at doses ranging between 0.2 ml/100 g and 0.4 ml/100 g to provide a relaxed muscle tone. Five to ten minutes later, the mice were anaesthetised with halothane and subsequently monitored until an anaesthetic plane, suitable for surgery, was attained. During surgery, mice were maintained on an anaesthetic plane with halothane and oxygen.

A 26-gauge, stainless-steel guide cannula, 0.80 cm in length, was unilaterally implanted in the IL prefrontal cortex using a David Kopf micromanipulator. All cannulae were implanted in accordance with the following stereotaxic coordinates: AP +2.0 mm anterior to Bregma, L +0.4 mm from the midsagittal suture and V –2.0 mm from a flat skull surface. The guide cannula was fixed in place with dental cement and jewellers' screws. Post-operatively, mice were individually housed and continued on an acetaminophen/drinking water regime for 3 days post-surgery. Animals were allowed a 2-week recovery period prior to experimentation.

### Drugs

NorBNI and pirenzepine were obtained from Sigma Chemical Co. (St. Louis, Mo.) and dissolved in artificial cerebrospinal fluid (CSF) each to a concentration of 10 nmol/0.5 µl. There were also two drug combinations: (1) norBNI-10 nmol/pirenzepine-10 nmol mix/0.5 µl and (2) norBNI-5 nmol/pirenzepine-10 nmol mix/0.5 µl. Artificial CSF was the vehicle.

### Intracerebral drug administration

Following a 2-week recovery period, mice were transferred from the main holding area to the testing laboratory and left undisturbed for 1 h prior to drug administration. The mice were randomly assigned to one of a vehicle or specific nanomole drug-dose treatment condition. Each mouse was lightly restrained and a 32-gauge injection cannula (connected using polyethylene tubing to a 10-µl Hamilton microsyringe) was inserted into the guide cannula. Each

drug concentration (0.5-µl volume) was infused over a 60-s period. The injection cannula remained in place for an additional 30 s to allow for maximum drug diffusion.

Immediately following drug infusion, each animal was returned to its home cage, brought into the testing room and left undisturbed for 5 min prior to behavioural evaluation. All testing was conducted during the morning phase of the light/dark cycle between the hours of 0800 hours and 1300 hours. All trials took place in a dimly illuminated chamber and were recorded by a ceiling-mounted Sony video camera that was linked to a Panasonic video monitor and VCR, and an IBM-compatible computer positioned outside the test chamber.

## Elevated plus maze

The elevated plus maze used in the present experiments was a modified version of a previously described apparatus (Lister 1987). The two opposing closed-arm runways were 30×5 cm and enclosed by 15-cm high walls on each side and ends. The two opposing open arms were also 30×5 cm with a 0.25-cm high Plexiglas edge on either side and at the end of each runway. The centre platform was 5×5 cm. The enclosed-arm walls were constructed with urethane-coated wood and lined with Plexiglas. The floor of the maze was covered with a thin layer of vulcanised black rubber to facilitate inter-trial cleaning. The apparatus was positioned 40 cm from the floor on a wooden stand hidden from view and the floor under the stand was covered with black cloth to reduce height cues.

## Y-maze

The Y-maze was a modified version of a previously described apparatus (Sarter et al. 1988; Hiramatsu et al. 1996; 1997). The three enclosed-arm runways were 30 cm long × 5 cm wide and enclosed by 15-cm high walls on each side and ends. The arms converged on an equilateral triangular centre platform (5×5×5 cm). The materials used to construct, and the appearance of, the Y-maze were identical to those for the elevated plus maze.

## Behavioural testing procedures

For each experiment, one-half of the animals from each group was tested in the first elevated plus-maze trial after a single injection followed by a no-injection trial 24 h later in week 1, then tested in the Y-maze after a second microinjection in week 2. The remaining animals were tested in reverse order (Y-maze in week 1 and elevated plus-maze trials 1 and 2 in week 2).

### *Elevated plus-maze anxiety test*

Each animal was challenged with one of a pre-trial vehicle or nanomole drug dose /0.5 µl microinjection in the IL cortex. Individual trials in the elevated plus maze were 5 min in duration and commenced by placing the animal in the centre hub, facing an

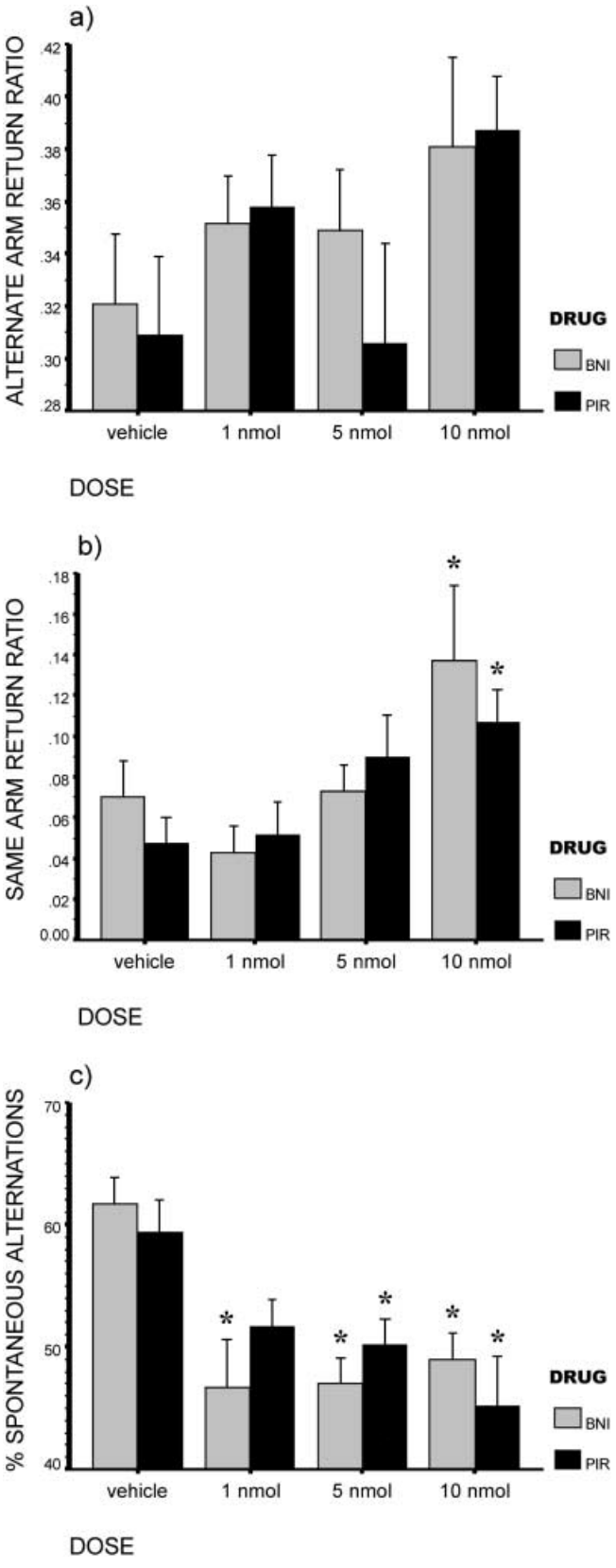


**Fig. 2a–c** Effects of infralimbic drug infusion interactions on rotational activity bias (alternate arm return ratio), attention (same arm return ratio) and spontaneous working memory (% spontaneous alternations) in the Y-maze. Values represent mean±SEM. *Grey bars* represent norBNI, *black bars* pirenzepine. *Significantly different than respective controls ($P$<0.05). The norBNI data were taken from Wall and Messier (2000a) and the pirenzepine data from Wall et al. (2001), with permission. Data sets were analysed together in two-factor ANOVAs to test for dose × drug interaction effects. There were no significant interaction effects on any measure

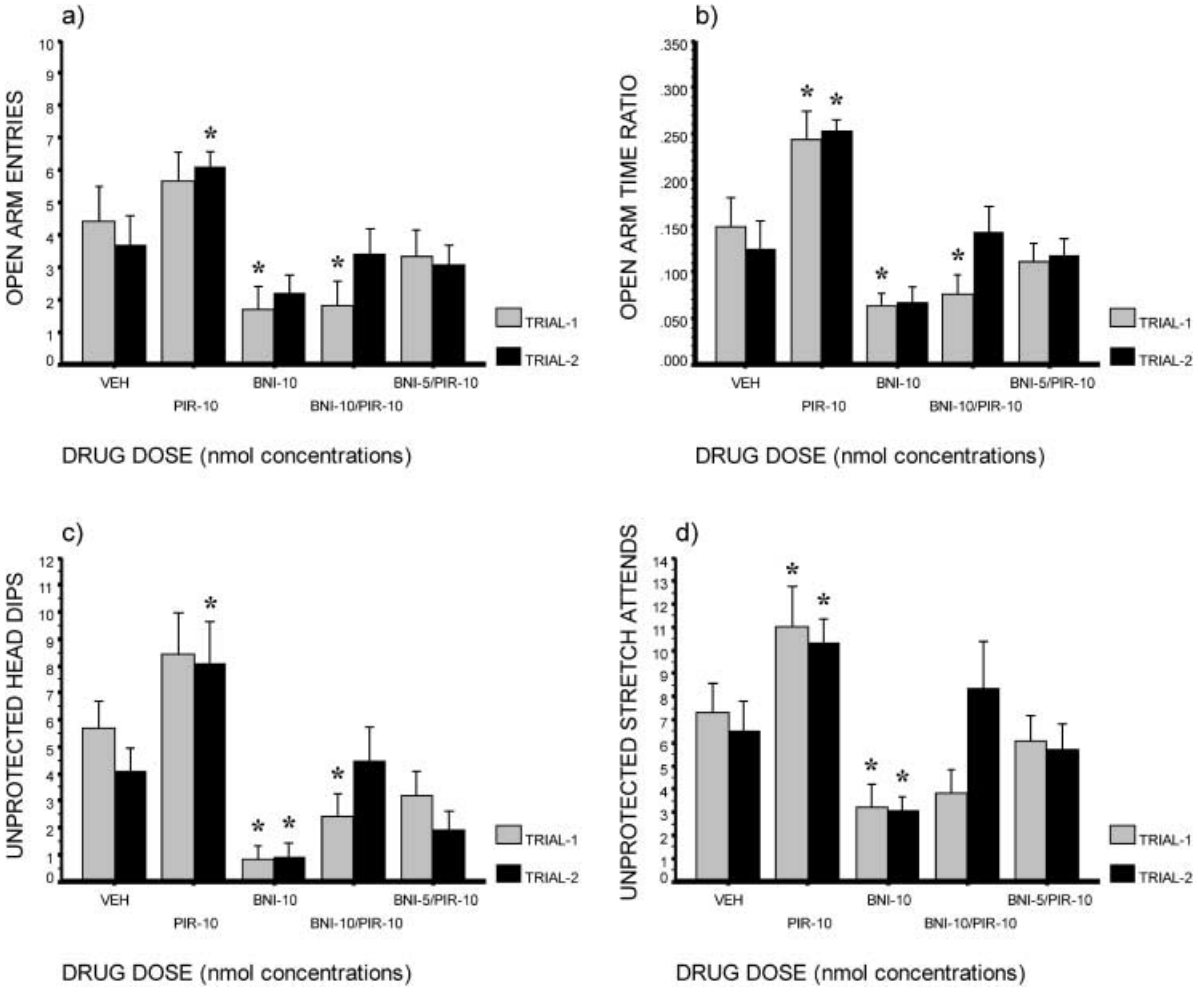


**Fig. 3a–d** Effects of pretrial-1 infralimbic drug infusions on elevated plus-maze behaviours. Values represent mean±SEM. *Grey bars* represent trial 1, *black bars* trial 2. *Significantly different than controls ($P<0.05$). **a** Open arm entries. **b** Open arm time ratio. **c** Unprotected head dips. **d** Unprotected stretch attends

open arm. A mouse was considered to have entered an arm when all four paws were positioned within an arm runway, or exited an arm (i.e., in the centre hub) when at least one paw was outside an arm threshold. After a 24-h delay, a second trial was conducted and recorded in the same manner as in trial 1, except there were no pre-trial drug injections. In this way, possible carry-over drug effects on aversive learning during trial-1 to aversive memory in trial 2 were evaluated in the second elevated plus-maze trial.

The anxiety-related behavioural indices in the elevated plus maze used in the present study have been validated and identified in several principal component analyses with rats and mice (Trullas and Skolnick 1993; Cruz et al. 1994; Rodgers and Johnson 1995; Fernandes and File 1996), as well as in our recent confirmatory factor analysis (Wall and Messier 2000b). We found that a two-factor model with seven indicator variables (cumulative open-arm entries, open-arm time ratio, unprotected head dips, unprotected stretch attends, closed-arm entries, closed-arm time ratio and closed vertical stretches) was the most parsimonious model that could unambiguously explain underlying constructs of plus-maze behaviour in two trials separated by a 24-h interval. Unprotected head dips were operationally defined as peering over the edge of an open arm with head, neck and shoulders, while out on an open-arm runway (Cole and Rodgers 1994). Unprotected stretch attends were operationally defined as the animal stretching its whole body forward while in an open arm and retracting to its original position without moving its hind paws (Cole and Rodgers 1994). Decreases in open entries, open time ratio, unprotected stretch attends and unprotected head dips were interpreted as anxiogenic indices of drug treatment relative to vehicle-treated animals, and decreases in closed entries, closed time ratio and closed vertical stretches were interpreted as reduced protected exploration activity.

*Y-maze spontaneous alternation memory and 'attention' tests*

Each animal was challenged with one of a pre-trial vehicle or drug dose/0.5 µl injection in the IL cortex. SAP was assessed by visually recording the spontaneous alternation behaviour of each mouse individually in the Y-maze in the same manner as previously described (Sarter et al. 1988; Itoh et al. 1994; Hiramatsu et al. 1997). A mouse was considered to have entered an arm when all four paws were positioned in the arm runway. Each mouse was allowed to freely explore the maze for 8 min. Alternations were operationally defined as successive entries into each of the three arms on overlapping triplet sets, and percentage spontaneous alternation performance (%SAP) was defined as the ratio of actual (total alternations) to possible (total arm entries–2) alternations ×100. Total entries were also scored as an index of ambulatory activity in the Y-maze (Hiramatsu et al. 1997).

In an effort to measure aspects of attention within spontaneous working memory, we scored alternate arm returns (AARs) and same arm returns (SARs) for each animal. If an animal went, for example, from arm A to arm B and back to arm A, one AAR was recorded. In addition, because some animals entered far fewer

**Table 1** Infralimbic drug effects on elevated plus-maze behaviour: pretrial-1 injections/elevated plus-maze trial 1. Values represent means±SEM. Time ratios represent the amount of time (s) in a section/300 s. *OE* open arm entries, *OTR* open time ratio, *UHD* unprotected head dips, *USA* unprotected stretch attends, *CE* closed arm entries, *CTR* closed time ratio, *CVS* closed vertical stretches. *Significantly different than vehicle ($P$<0.05)

| Behaviour | Vehicle | Pirenzepine 10 nmol | BNI 10 nmol | BNI 10/ Pirenzepine 10 | BNI 5/ Pirenzepine 10 | $F_{4,46}$ | $P$ value |
|---|---|---|---|---|---|---|---|
| OE | 4.4±1.0 | 5.7±0.8 | 1.7±0.4* | 1.8±0.4* | 3.3±0.6 | 5.8 | 0.001 |
| OTR | 0.149±0.031 | 0.243±0.029* | 0.063±0.013* | 0.076±0.021* | 0.111±0.021 | 8.7 | 0.000 |
| UHD | 5.7±1.0 | 8.4±1.5 | 0.8±0.3* | 2.4±0.8* | 3.2±0.9 | 9.0 | 0.000 |
| USA | 7.3±1.3 | 11.0±1.8* | 3.2±0.8* | 3.8±1.1 | 6.1±1.1 | 5.9 | 0.001 |
| CE | 11.5±1.5 | 11.9±1.5 | 12.9±1.3 | 12.5±1.2 | 12.7±1.0 | 0.2 | NS |
| CTR | 0.389±0.036 | 0.422±0.037 | 0.396±0.043 | 0.405±0.046 | 0.412±0.023 | 0.1 | NS |
| CVS | 16.8±2.1 | 22.9±3.8 | 18.5±2.6 | 17.0±2.4 | 16.5±1.6 | 1.1 | NS |

**Table 2** Carry-over drug effects on trial-2 elevated plus-maze behaviour 24 h later (no injection). Values represent means±SEM. Time ratios represent the amount of time (s) in a section/300 s. *OE* open arm entries, *OTR* open time ratio, *UHD* unprotected head dips, *USA* unprotected stretch attends, *CE* closed arm entries, *CTR* closed time ratio, *CVS* closed vertical stretches. *Significantly different than vehicle ($P$<0.05)

| Behaviour | Vehicle | Pirenzepine 10 nmol | BNI 10 nmol | BNI 10/ Pirenzepine 10 | BNI 5/ Pirenzepine 10 | $F_{4,46}$ | $P$ value |
|---|---|---|---|---|---|---|---|
| OE | 3.7±0.8 | 6.1±0.4* | 2.2±0.5 | 3.4±0.7 | 3.1±0.4 | 6.2 | 0.000 |
| OTR | 0.126±0.027 | 0.254±0.024* | 0.067±0.015 | 0.143±0.028 | 0.118±0.018 | 8.5 | 0.000 |
| UHD | 4.1±0.9 | 8.1±1.6* | 0.9±0.4* | 4.5±1.2 | 1.9±0.7 | 7.8 | 0.000 |
| USA | 6.5±1.3 | 10.3±0.8* | 3.1±0.6* | 8.4±1.8 | 5.8±0.9 | 5.2 | 0.002 |
| CE | 8.5±0.9 | 13.7±1.2* | 14.3±0.7* | 11.4±1.2* | 12.3±1.0* | 5.1 | 0.002 |
| CTR | 0.329±0.032 | 0.418±0.032 | 0.482±0.028* | 0.404±0.048 | 0.435±0.027* | 2.6 | 0.045 |
| CVS | 13.6±1.5 | 23.4±2.3* | 25.8±1.6* | 18.2±2.3 | 22.0±1.5* | 6.7 | 0.000 |

arms than others, we reasoned that the AAR score would be biased. Thus, AAR was expressed as a ratio of alternate arm returns/total arm entries ×100. In addition to conceivably estimating some aspects of attention in the Y-maze, AAR also provides a measure of locomotor rotational bias in the Y-maze. Since there is some suggestion that unilateral intracerebral drug infusions oftentimes bias an animal to continuously turn in the opposite direction of the cannulated hemisphere, the AAR measures the opposite of this effect. Thus the higher the AAR ratio, the less directionally biased an animal would be.

SAR was also scored as a ratio over total arm entries ×100. SARs measured cumulative returns into the same arm (e.g., if an animal leaves arm A and then returns into arm A, 1 SAR was recorded, and so on). We reasoned that the SAR ratio might specifically tap into the degree of attentional difficulties the animal might be experiencing in the Y-maze. In this respect, if SAP taxes active retrograde working memory processing such that the animal must maintain an ongoing record of most recently visited arms, and attentional mechanisms continuously update such a record, then the SAR ratio should conceivably estimate some aspects of attentional dysfunction within active working memory performance in the Y-maze. Preliminary dose–response data from our previous norBNI and pirenzepine studies provided support to measure both indices in the present study (see Results and Fig. 2 for dose–response effects of norBNI and pirenzepine on AAR and SAR).

## Histology

Mice were sacrificed with an overdose of sodium pentobarbital and perfused intracardially with 10 cc 0.9% saline, followed by 10 cc 10% formalin. Mice were decapitated, head caps removed, brains excised from the cranial cavity and stored in a 10% formalin/30% sucrose solution for at least 1 week before histological analysis. Mouse brains were individually blocked and frozen on a cryostat. Coronal sections (30 µm) were taken through the rostral–caudal extent of each cannula placement. Brain sections were mounted, stained with cresyl violet and examined under a microscope. The distribution of cannulae tip placements in the IL cortex were mapped on schematic representations employing the mouse brain atlas of Franklin and Paxinos (1997). Only those subjects with correct cannulae tip placements in the IL cortex were included in the present analyses: within the rostral–caudal range +1.94 mm to +2.1 mm anterior to Bregma, the medial–lateral range +0.1 mm to +0.7 mm lateral to the midsagittal suture and the dorsal–ventral range –2.5 mm to –3.25 mm ventral to a flat skull surface. The included cannulae placements are similar to those depicted in previous reports (Wall and Messier 2000a, 2000c). Two mice from the vehicle, three from the pirenzepine-10 nmol, two from the norBNI-10 nmol and two from the norBNI-10/pirenzepine-10 nmol groups were excluded due to placements outside this circumscribed region. In all, 10 vehicle, 9 pirenzepine-10 nmol, 10 norBNI-10 nmol, 10 norBNI-10 nmol/pirenzepine-10 nmol, and 12 norBNI-5 nmol/pirenzepine-10 nmol ($n$=51) mice were included in the analyses.

## Statistical analyses

The pirenzepine and norBNI data from two previous studies (Wall and Messier 2000a; Wall et al. 2001) were analysed together in two-factor ANOVAs to determine dose × drug interaction effects ($\alpha$=0.05) on four elevated plus-maze anxiety indices [open-arm entries (OE), open-arm time ratio (OTR), unprotected head dips (UHD), unprotected stretch attends (USA)] and three Y-maze indices (AAR, SAR, %SAP). The behavioural data from the present elevated plus-maze experiment were analysed using one-way ANOVA for trials 1 and 2 ($\alpha$=0.05). Post-hoc LSD tests were conducted for significant treatment effects relative to control means ($\alpha$=0.05). Y-maze total entries, %SAP, AAR and SAR were also analysed using one-way ANOVA ($\alpha$=0.05). Post-hoc LSD tests were conducted for significant treatment effects relative to control means ($\alpha$=0.05).

## Results

Dose–response interaction analyses of our previous norBNI and pirenzepine data revealed that there were significant dose × drug interaction effects on open-arm entries in trial 1 ($F_{3,62}$=5.4, $P$<0.005) and trial 2 ($F_{3,62}$=10.2, $P$<0.001), open-arm time ratio in trial 1 ($F_{3,62}$=5.9, $P$<0.002) and trial 2 ($F_{3,62}$=9.9, $P$<0.001), unprotected head dips in trial 1 ($F_{3,62}$=5.8, $P$<0.002) and trial 2 ($F_{3,62}$=6.4, $P$<0.002), and unprotected stretch attends in trial 1 ($F_{3,62}$=6.8, $P$<0.001) and trial 2 ($F_{3,62}$=10.5, $P$<0.001) (Fig. 1). These results provided evidence in support of the hypothesis that simultaneous kappa and M1 receptor blockade should exert counteractive effects on aversive learning and memory, and anxiety in the elevated plus maze.

Further analyses of our previous norBNI and pirenzepine data revealed that there were no effects of drug or dose on the AAR ratio (Fig. 2a). These data suggest that unilaterally microinjecting either drug produced negligible directional or perseverative biases in our previous studies, thus mixed unilateral drug injections should also minimally bias locomotor activity.

There were, however, simple effects of dose on the SAR ratio:

- norBNI ($F_{3,26}$=3.6, $P$<0.03): the 10-nmol norBNI dose increased SAR relative to vehicle controls ($P$<0.05)
- Pirenzepine ($F_{3,36}$=3.0, $P$<0.05): the 10-nmol pirenzepine dose increased SAR relative to vehicle controls ($P$<0.05)

There were no interaction effects (Fig. 2b). These data indicated that the 10-nmol dose of each drug disrupted some aspects of attention in our previous studies; thus, mixed 10-nmol drug injections might produce additive effects on some aspects of attention in the Y-maze.

There were also simple effects of dose on %SAP norBNI ($F_{3,26}$=8.8, $P$<0.001), the 1-, 5- and 10-nmol norBNI doses disrupted %SAP relative to the vehicle controls ($P$<0.05); pirenzepine ($F_{3,36}$=4.9, $P$<0.01), the 5-nmol and 10-nmol pirenzepine doses disrupted %SAP relative to controls ($P$<0.05). There were no interaction effects (Fig. 2c).

### Elevated plus-maze anxiety tests – trials 1 and 2 (present data)

The behavioural data along with associated ANOVAs and post-hoc LSD tests for trials 1 and 2 are summarized in Table 1 and Table 2, respectively. Figure 3 and Fig. 4 depict results from both elevated plus-maze trials for comparison purposes.

### Y-maze spontaneous alternation memory, AAR and SAR

One-way, between-subjects ANOVA showed that no drug treatment influenced Y-maze total arm entries.

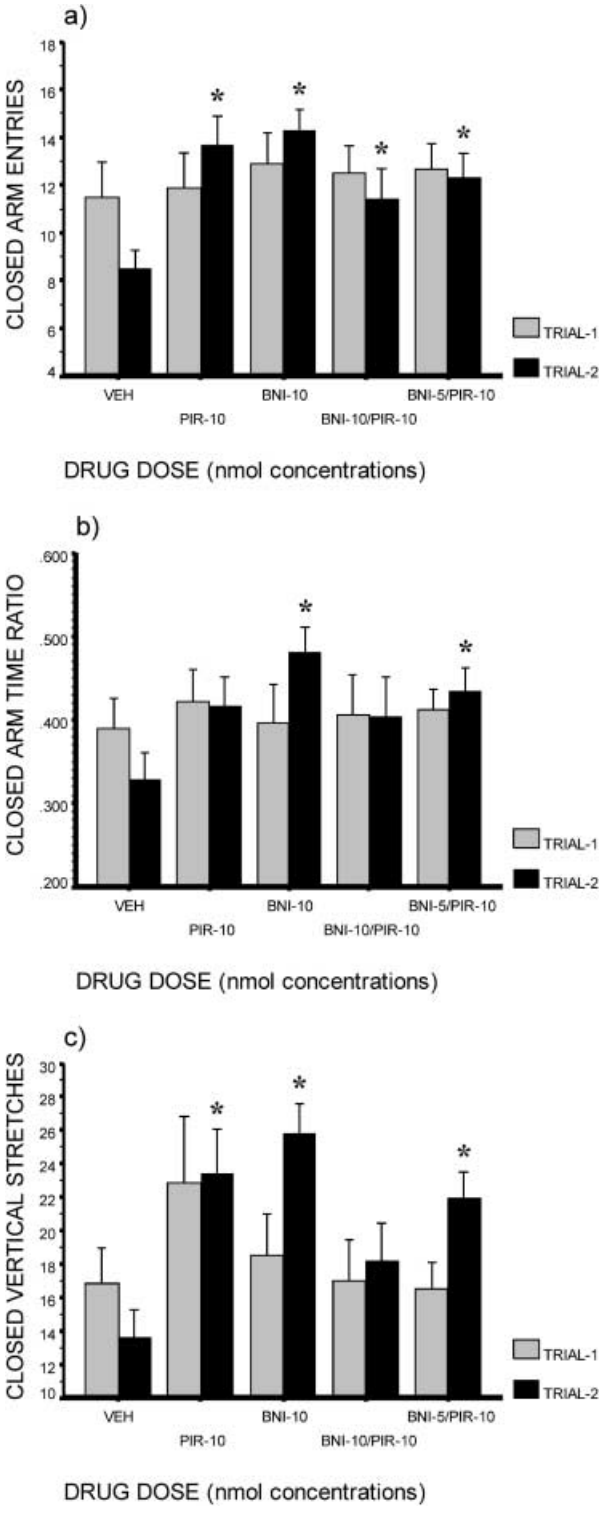


**Fig. 4a–c** Effects of pretrial-1 infralimbic drug infusions on elevated plus-maze behaviours. Values represent mean±SEM. *Grey bars* represent trial 1, *black bars* trial 2. *Significantly different than controls ($P$<0.05). **a** Closed arm entries. **b** Closed arm time ratio. **c** Closed vertical stretches

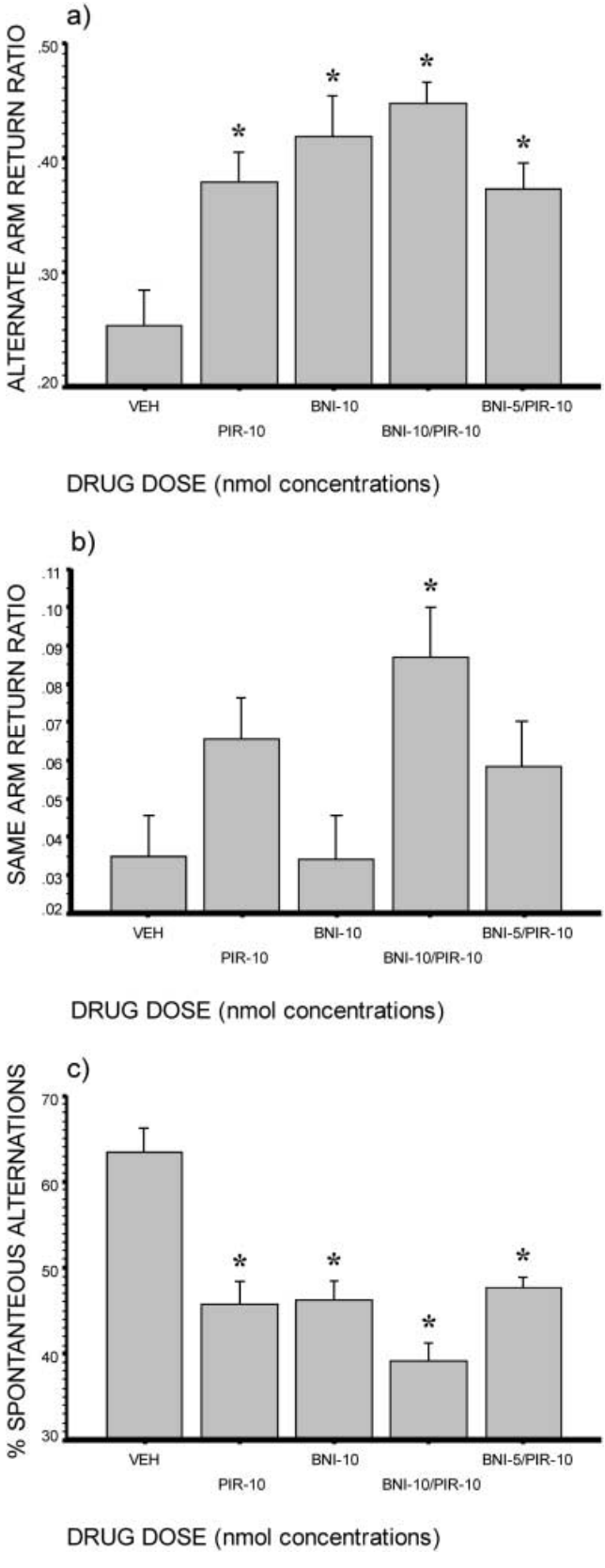


**Fig. 5a–c** Effects of infralimbic drug infusions on Y-maze indices. Values represent mean±SEM. *Significantly different than vehicle controls ($P$<0.05). **a** Alternate arm return ratio. **b** Same arm return ratio. **c** Percentage spontaneous alternations

Drug treatment did, however, influence AAR ($F_{4,46}$=8.3, $P$<0.001), SAR ($F_{4,46}$=2.9, $P$<0.05), and %SAP ($F_{4,46}$= 18.6, $P$<0.001) in the Y-maze. Post-hoc LSD tests revealed that all four drug treatments increased AAR ($P$<0.05), the norBNI-10 nmol/pirenzepine-10 nmol mix infusions increased SAR ($P$<0.05), and %SAP was disturbed by all four drug treatment levels ($P$<0.05) relative to vehicle-treated control mice (Fig. 5).

These data lend some support to the notion that the norBNI-10 nmol/pirenzepine-10 nmol drug mix produced additive disruptive effects on SAP and some aspects of attention related to active working memory in the Y-maze.

## Discussion

The results from previous and the present experiments support the involvement of IL M1 and kappa receptor interactivity in the concurrent regulation of anxiety-like behavioural responding and associative aversive learning and memory in CD-1 mice. Moreover, the present data indicate that IL M1 and kappa receptors may additively influence attentional mechanisms related to active working memory. Although this interpretation of the present results is compelling, the data are preliminary thus evidence supporting our hypothesised relationships among the psychological constructs of anxiety, aversive learning and memory, attention and active working memory are subsequently only beginning to emerge. We can nevertheless speculate further upon possible neurochemical substrates in the vmPFC that might concurrently influence these psychological constructs and, indeed, possible relationships between constructs.

First, consistent relationships between anxiety-like responding and aversive learning and memory in the elevated plus maze have emerged throughout our pharmacological studies in the vmPFC (Wall and Messier 2000a, 2000c; Wall et al. 2001). Whereas anxiolytic drugs conceivably disrupted aversive associative learning during trial 1 and memory for such learning during trial 2 in the elevated plus maze, anxiogenic drugs conceivably facilitated aversive learning and memory. These observed relationships make intuitive sense and support the hypothesis that the vmPFC concurrently modulates both cognitive/behavioural constructs.

Second, we can also speculate that associative aversive learning and memory coupled with the evoked behavioural patterns evident among the mice placed in the elevated plus maze result from active attentional and working memory/set processing in the vmPFC (Wall and Messier 2001). The idea here is that active associative aversive learning and memory, and attentional processing related to active working memory/set, likely involve overlapping neural circuitry in the vmPFC. Intuitively, active working memory and active working cognitive/behavioural set involve related attentional processes in vmPFC (Fuster 1995, 1997; Wall and Messier 2001). Indirect evidence in support of this hypothesis stems

from consistent demonstrations that both kappa and M1 drug infusions in the IL cortex influence neural activity related to the expression of anxiety-related behaviour, as well as attentional aspects of active working memory during spontaneous exploration.

Third, considering the data demonstrating that IL norBNI or pirenzepine infusions enhanced or reduced, respectively, anxiety-like responding in the elevated plus maze and similarly reduced spontaneous working memory performance in the Y-maze, we speculated that kappa and M1 receptors might interact in the vmPFC to concurrently influence anxiety and attentional mechanisms related to active working memory processing. Clearly, when we re-analysed our previous norBNI and pirenzepine dose–response data together using two-factor dose × drug ANOVAs, consistent interactive effects were statistically shown on all anxiety-related indices in both elevated plus-maze trials. Moreover, further analyses of our previous norBNI and pirenzepine data showed that the 10-nmol dose of each drug disrupted some aspects of attention (SAR ratio) in the Y-maze.

Fourth, we reasoned that since statistical interactions are only suggestive of receptor interactivity in the concurrent modulation of anxiety and associative learning and memory, then mixed norBNI/pirenzepine drug infusions should more distinctly demonstrate interactive drug effects on anxiety-related indices and associative learning and memory. This was indeed the case. Although the norBNI-10 nmol/pirenzepine-10 nmol mixed drug injections exerted some (net) anxiogenic effects on anxiety-related behaviour during trial 1 in the elevated plus maze, aversive learning must not have occurred during trial-1 owing to the demonstration that there were no carry-over mixed drug effects on anxiety-related behaviour during trial 2. Thus, the anxiogenic effects of 10 nmol norBNI infusions alone that are normally coupled with enhanced aversive learning during trial 1 appeared to be counteracted by the 10-nmol pirenzepine dose in the mixed drug condition. In fact, the norBNI-10 nmol/pirenzepine-10 nmol group actually showed some increased unprotected exploration activity during trial 2 relative to controls in the present study. Moreover, by lowering the norBNI dose to 5 nmol in the norBNI-5 nmol/pirenzepine-10 nmol mixed drug condition, the trial-1 anxiogenic effects of the norBNI-10 nmol/pirenzepine-10 nmol drug infusions were no longer evident, such that the norBNI-5 nmol/pirenzepine-10 nmol infusions had no net effects on any of the four anxiety-related indices or associative learning and memory in the elevated plus maze.

Fifth, since the norBNI-10 nmol/pirenzepine-10 nmol mixed drug infusions additively disrupted Y-maze SAP to a greater extent than either norBNI-10 nmol or pirenzepine-10 nmol infusions alone in the present or previous studies (Fig. 2c and Fig. 5c), then we can speculate that concurrent kappa/M1 receptor inhibition likely exerted additive disruptive effects on attentional processing during Y-maze exploration. The present SAR data partially support this hypothesis insofar as the SAR ratio can be argued to be a valid index of attentional processing related to active working memory performance in the Y-maze (see Methods).

We can now speculate on possible neurochemical explanations of the differential effects norBNI and pirenzepine exerted on relationships between anxiety and active working memory. Since it is well known that acute dopamine (DA) activation in the vmPFC has been primarily associated with aversive events and anxiety-like responses (Broersen et al. 1995a), and working memory disruption (Murphy et al. 1996a, 1996b; Arnsten and Goldman-Rakic 1998), then it appears reasonable to suggest that IL norBNI infusions may have enhanced an already-activated DA release to enhance anxiety and reduce spontaneous memory performance (Wall and Messier 2000a). We can also suggest that M1 receptors in the vmPFC might primarily influence prospective attentional processing associated with working memory, working set and anxiety. This hypothesis is in keeping with Sarter and Bruno's proposal that prolonged cholinergic transmission disruption in the PFC disrupts attentional processing (Sarter and Bruno 1999). Moreover, oftentimes working memory disrupting effects of muscarinic antagonist infusions in the vmPFC have been reported as disruptive effects on attention or response inhibition (Dunnett 1990; Broersen et al. 1995b; Herremans et al. 1996, 1997).

We can further speculate that the differential correspondence between active working memory and anxiety following norBNI and pirenzepine infusions may have occurred through bi-directional interactivity with glutamate (GLU) afferents in the vmPFC. Indirect evidence in support of this hypothesis stems from in vivo microdialysis data showing that DA D1 receptor activation in the vmPFC reduced GLU release in rats (Abekawa et al. 2000). These investigators suggested that prolonged dopaminergic hyperactivity in the vmPFC may lead to prefrontal GLU hypofunction (Abekawa et al. 2000). Moreover, M1 receptor activation following McN-A-343 infusions in the vmPFC enhanced GLU concentrations in rats, and these GLU-releasing effects were blocked by pirenzepine infusions (Sanz et al. 1997). Thus, it is tempting to suggest that IL norBNI infusions might indirectly impair active working memory performance through DA effects on GLU activity, and IL pirenzepine infusions might directly impair attentional aspects related to active working memory through direct effects on GLU afferent activity in the vmPFC. These hypotheses are highly speculative but, nevertheless, warrant further investigation.

It is interesting that many of the cognitive aspects of Alzheimer's, schizophrenia and attention deficits are strongly linked to dopaminergic and cholinergic dysfunction in the mPFC (Knable and Weinberger 1997; Okubo et al. 1997; Ernst et al. 1998; Lawrence and Sahakian 1998; Bymaster et al. 1999; Cosford et al. 2000; Faraone et al. 2000; Goldman-Rakic et al. 2000). These data underscore the need to not only understand the link between anxiety, attention and working memory

in the vmPFC, but also how cholinergic and dopaminergic systems might interact to link these cognitive/behavioural constructs in this prefrontal associative network.

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