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IN CONFIDENCE

SCIENTIFIC RESEARCH GRANT REPORT FORM

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This form should be completed in typescript or black ink. The submission of a Scientific Report is regarded by the Council as an essential requirement of their Grant Schemes. Parts 1-5 should correspond with the information contained in the award letter. Any changes must be clearly identified and explained in the text of the report.
Send this form and your detailed report (see Section 19) with (two) copies of each to reach the MRC within 3 months of the end of the research grant. Failure to do so will result in financial penalties. This report may be used as part of a Progress report or when requesting a Renewal or Extension. Investigators are asked to restrict their comments to the spaces provided on this form.

1. GRANT NUMBER:	2. GRANT PERIOD:	3. TYPE OF REPORT:
G 9616330N TYPE OF GRANT: Small Project Grant	START DATE: 01 / 09 / 97 END DATE: 31 / 08 / 98	PROGRESS RENEWAL EXTENSION FINAL Final

4. INVESTIGATOR(s):	INSTITUTION(s) / AUTHORITY
Dr Judith Pratt	University of Strathclyde DEPARTMENT WHERE WORK DONE Physiology & Pharmacology

5. TITLE OF INVESTIGATION:

The neurochemistry of benzodiazepine withdrawal; a focus on glutamate and dopamine release using microdialysis.

6. OBJECTIVES OF THE RESEARCH:
List the main objectives as stated in the original proposal in order of priority

PROMPT
Scientific aims & any plans for the application / exploitation as stated when funding originally sought.

The overall objective was to test the hypothesis that changes in glutamate transmission are important in benzodiazepine withdrawal.

Specific objectives
Determine if withdrawal from diazepam is asociated with an increase in glutamate overflow in the nucleus accumbens and the anterior thalamus of the Papez circuit.
Investigate the acute, chronic and withdrawal effects of diazepam upon nucleus accumbens dopamine transmission, and to establish whether these changes can be correlated with changes in amino acid transmission.

7. ACHIEVEMENTS OF THE RESEARCH:
Describe the extent to which the objectives of the research have been achieved and relate the significance of the scientific advances/insights achieved to recent work in the field.

Identify important results and relate to general developments of the field. Please explain any changes in objectives during the study.

The overall objectives of the research have been largely achieved but the methodological approaches employed have differed from that in the original application. An additional objective, namely to investigate a role for AMPA receptors in the reinforcing properties of diazepam has also been achieved.
We have demonstrated that withdrawal from chronic low dose benzodiazepine treatment produces regionally selective increases in AMPA receptor binding consistent with our hypothesis of an increase in glutamatergic transmission during drug withdrawal. This is a novel finding and provides a neurochemical explanation for recent behavioural evidence implicating altered glutamatergic transmission during benzodiazepine dependence as well as accounting, in part, for the regional changes in neural activity demonstrated in our previous work.
Another important finding from our studies was that the AMPA receptor antagonist GYK152466 blocks the acquisition of diazepam-induced place preference. This supports the accumulating evidence (with other drugs of abuse) of a role for glutamate receptors in drug reinforcement.
Our microdialysis studies have shown that benzodiazepines reduce DA concentration in the nucleus accumbens but that a glutamate antagonist does not.

8. PROGRESS OF THE RESEARCH:
(i) Outline the methodology used in the research.

Examples (design) tissues/cells, techniques/ approaches, measurements/outcomes.

(a) *In vivo* microdialysis
(b) Quantitative receptor autoradiography
(c) Conditioned place preference

(ii) Was there any significant change in the research work or programme of work compared with the original proposal. YES/NO

If YES give reasons for changes. i.e. Did the research proceed as expected and on time? If NO give details. Were there any circumstances which aided or impeded the progress of the research? If YES, explain the steps you took to overcome them. Examples of problems could include difficulties in recruitment of staff, late delivery of equipment and malfunction of equipment.

The start of the project was delayed since the original named worker accepted a 5 year post. Recruitment of staff with appropriate microdialysis experience proved difficult and so it was decided to appoint a graduate with an excellent academic record instead.
The *in vivo* microdialysis method for dopamine has been established. However, in light of the referees comments we have explored alternative analytical methods for glutamate. To this end, we have revised the methodological employed. This necessitated purchasing a fluorescence detector, an additional HPLC pump and new software. Some of the equipment component of the grant was used for this purpose. An autosampler was not deemed essential and so these items were purchased in its place with some additional financial support from the Department. Progress on this aspect of the project has been delayed because the additional monies were not available until June 1998 and in July 1998 we had to cease animal work because of relocation to a new building. Nevertheless very good research progress has been made. This has involved utilising the techniques of quantitative receptor autoradiography and conditioned place preference.

9. FURTHER RESEARCH:

(i) Has the research led to further investigations or collaborations which have led to other applications to the MRC or others? YES/NO

If YES, give details of the outcome. List grant applications giving dates and grant reference. Indicate value of any grants awarded. Give details of the outcome. List non - MRC grant applications, collaboration in EC research programmes and industrially supported work.

A collaboration has developed with [redacted]

10. RESOURCES AND PEOPLE:

i) Detail all grants/contracts/major increases funded by the Host Institution which arose through this research proposal.

Give title, funder, tenure and value.

Grant for equipment; 6K. Salary for Graduate Teaching Assistant, Sept 98-Aug 01; 36K.

ii) List the staff employed directly on the grant. List any other research fellows and research students associated with the research project and their sources of support.

Give the grade and period of working on the project for all staff. Indicate source of support for any research fellows and research students (include MRC and all sources of support). Also indicate the degrees for which students are enrolled.

Direct employee
Ms Claire Allison, Sept 97-Aug 98, 1A Grade. Registered for PhD.
Associated with project
Dr Alex Gray, Postgraduate Fellow, University of Strathclyde, 1996.

(iii) Describe what staff development and training has resulted from this project.

Detail any training or development benefits to staff employed on the grant including PhD awards that have arisen from the research.

Claire Allison is registered for a PhD. She has presented a seminar of her work to the Department and attended the British Association for Psychopharmacology meeting (July '98). She will present her findings at the local group of the British Neuroscience Association (December '98) and at the British Pharmacological Society (January '99). Claire has spent one week in Professor Stephen's laboratories in Sussex as part of a research collaboration. Her career development continues in her current post as a Graduate Teaching Assistant. She is involved in various aspects of undergraduate teaching (laboratory classes and tutorials) and will attend training courses for new lecturers.

11. COLLABORATION:

Did any other person or institution / organisation collaborate in the research.

Give details and describe the extent of all the collaborations anticipated in your proposal or which emerged during the collaboration (e.g. include grants from or formal collaborations with industry, exchanges of staff, materials or results arising from the research). Indicate the extent to which arrangements between the awardholders and collaborators have been realised and whether the Collaborators contribution differed from anticipated.

A collaboration has been initiated with [redacted]
This has resulted in exchange of staff and materials. This work was not directly related to the grant but to an extension of the work relating to repeated withdrawal of benzodiazepines in which both parties share an interest.

12. FACILITIES:

Give details of any facility used for this research.

Name of Facility	Time Allocated	Time Used
-		

Identify use of international and national facilities and facilities within HEIs. E.g. CCLRC, SRS etc.

13. PUBLIC AWARENESS OF SCIENCES (PAS):

What activities have been undertaken to promote the public awareness of the scientific results arising from this research, and did you refer to MRC support.

-

Give details of work/activity undertaken and an indication of the costs. Include details of media coverage, exhibitions, school links, articles in popular journals etc. Please detail any significant publicity.

14. EXPENDITURE:

(i) Has expenditure exceeded or fallen short of the total sum awarded under a particular heading? YES/NO

Additional consumables (= 2K) for radiochemicals were required because of the decision to include receptor autoradiography experiments in the research programme. Additional monies for equipment (= 6K) were made available from the Department.

If YES, explain the unexpected patterns of expenditure.

(ii) Did you need to vire any funds between heads, if so why?

Some monies (= 2K) were vired from the equipment budget to the consumables budget to support the autoradiography studies.

15. PUBLICATION AND DISSEMINATION OF RESULTS:

(i) List titles of papers and internal reports etc. arising from the research (including items in preparation) and did you refer to MRC support.

List publications, (in refereed journals and others) detailing authors, underlining the names of authors funded by this grant, date, title, journal volume, page no's, (where known), conference proceedings, book chapters etc. . Include publications which have arisen through these collaborations. Please detail any significant publicity.

Gray A, **Allison C & Pratt JA** (1998) A role for AMPA receptors in diazepam-induced conditioned place preference. Neuroreport (submitted).

Allison C & Pratt JA (1999) Regional alterations in [^{3}H]AMPA binding in rat brain after chronic diazepam treatment. To be presented at Brit Pharm Soc January 1999.

Allison C & Pratt JA (1998) Changes in glutamatergic transmission after chronic diazepam treatment. To be presented at British Neuroscience Association, Glasgow Group Meeting, December 1998.

(ii) Has any data been lodged in a public access database. YES/NO

If YES, give details.

No

16. EXPLOITATION OF RESULTS:

(i) Who are the likely beneficiaries of the research and have you disseminated any of the results to the User Communities (NHS, Industry etc.)

E.g. other researchers, business and commerce, local or central government and other users.

Other neuroscience researchers

(ii) Record anything patentable / commercially exploitable arising from the research, in the short, medium or long term?

Give details and describe what arrangements have been made or are planned for exploitation of the results.

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(iii) What are the implications for improving health and health care or quality of life in the short, medium or long term and what progress is being made towards exploiting these opportunities.

Refer to any actual or potential application or exploitation of research or relevance to Government Department priorities.

Potential, in the long term, for glutamate receptor antagonists in the treatment of drug dependence.

(iv) Record any increased collaboration with existing or new industrial commercial partners and any new sponsorship, funding for basic/strategic research.

This part of the report is particularly relevant to holders of ROPAs.

New commercial venture with Yoshitomi Pharmaceutical Industries Ltd for schizophrenia research (not directly related to above grant) has resulted in the establishment of the Yoshitomi Research Institute for Neuroscience in Glasgow (YRING) of which I am Co-Director. This is a multimillion pound initiative.

18. DETAILED REPORT ATTACHED: You may also attach:	
(i) a report of not more than six sides* of a A4 typescript (point size 12) with a list of references. It should outline the scientific and / or technological achievements of the research expanding as necessary on the answers provided above.	Do not submit lengthy internal reports or PhD theses. Copies of key publications arising directly from the investigation should be appended, but are not acceptable as a substitute for any part of this report.
(ii) a separate summary (maximum of one A4 page) suitable for publication describing the achievements made on the Research Grant.	Include title, investigators, institutions and the name of a person whom readers should contact.

19. SIGNATURES	SIGNATURES AND DATES
INVESTIGATOR(s):	[redacted] 25.11.98.
HEAD(S) OF DEPARTMENT(S):	[redacted]
ADMINISTRATIVE AUTHORITY; POSITION HELD:	[redacted] 26/11/98 MANAGER RESEARCH GRANTS AND CONTRACTS

Report on G9616330N

The neurochemistry of benzodiazepine withdrawal; a focus on glutamate and dopamine release using microdialysis.

Grant holder: Dr J. Pratt
Research Assistant appointed on grant: Ms C. Allison

The overall objective of the research programme was to test the hypothesis that changes in glutamate transmission are important in benzodiazepine withdrawal.
The original aim was to employ the technique of *in vivo* microdialysis to explore changes in glutamate release in specific brain regions during withdrawal from diazepam. However, we decided that it would also be very important to evaluate whether there were regionally selective increases in glutamate receptor binding during drug withdrawal as determined by quantitative receptor autoradiography. In addition, as part of our broader interests in the area we have also examined whether glutamate receptors are involved in the reinforcing properties of diazepam.

The research programme has therefore utilised three different experimental approaches; quantitative receptor autoradiography, in vivo microdialysis and conditioned place preference. The results from each of these are discussed in turn.

Glutamate receptor binding studies

In previous work, we have found that changes at the level of the $GABA_A$ receptor do not provide an adequate explanation for the changes in neural activity in circuits recruited during chronic benzodiazepine treatment (see Pratt et al 1998; Pharmacol Biochem Behav 59, 925-934). Alternative neurochemical mechanisms therefore need to be considered. The hypothesis that chronic enhancement of GABAergic activity by chronic diazepam treatment causes a compensatory increase in glutamatergic excitatory mechanisms was therefore the focus of this study. Very good progress has been made in this part of the research programme. Regional changes in glutamate receptor properties were assessed by investigating whether or not withdrawal from chronic diazepam treatment produced increases in AMPA receptor binding as determined by quantitative receptor autoradiography.

Experiments were performed on male Long Evans hooded rats which received daily injections of diazepam (5 mg/kg i.p.) for 14 and 28 days or repeated vehicle injections. Twenty four hours after the last treatment, rats were killed and the brains removed and sectioned at 8 levels for autoradiography. [^{3}H] AMPA autoradiography was performed using a modification of a previous method (Dev and Morris (1994) J. Neurochem 63, 946-952) with 10 μM quisqualic acid being used to determine non specific binding. Withdrawal from 28 days diazepam treatment resulted in regionally selective increases in AMPA binding. Significant increases occurred in the CA2 field of the hippocampus and in the frontal cortex. These changes were also apparent in the hippocampus following 14 days of diazepam treatment. There were no significant changes in other brain areas examined including other limbic structures. These data provide preliminary evidence which supports the hypothesis of enhanced glutamatergic mechanisms following chronic benzodiazepine treatment. The results also provide in part, an explanation for the regionally selective increases in neural

activity demonstrated in our previous work. We are currently substantiating these studies by examining whether CNQX binding displays similar regional increases in binding. This ligand binds to both high and low affinity forms of the AMPA/kainate receptor whereas under the experimental conditions employed for [^{3}H] AMPA only high affinity sites are labelled.

This aspect of the research programme will be presented at the Glasgow group meeting of the British Neuroscience Association (Dec 1998) and the British Pharmacological Society Meeting (Jan 1999). A copy of the Abstract is attached.

A further development during the course of this grant has been the development of a collaboration with [redacted] This is concerend with examining the effects of repeated withdrawal from diazepam upon binding to glutamate receptor subtypes.

Neurotransmitter release studies

One objective of the original investigation were to determine if withdrawal from diazepam was associated with increases in glutamate overflow in the nucleus accumbens and the anterior thalamus of the Papez circuit using *in vivo* microdialysis. In addition, we planned to investigate the acute, chronic and withdrawal effects of diazepam upon nucleus accumbens dopamine transmission, and establish whether these changes could be correlated with changes in amino acid transmission.

Progress on this aspect of the project has been subject to some delays due to a variety of factors. Firstly, the original named person on the grant took up a three year position in preference to this post. Secondly no high calibre persons with microdialysis expertise were forthcoming. This led to the appointment of a graduate, Ms C. Allison, who naturally required training in the research techniques. Thirdly, we decided to thoroughly evaluate the analytical methodologies employed for glutamate microdialyis as a result of the refereees comments on the grant. During this time we decided that valuable information could be gained if we also conducted an analysis of glutamate receptors by autoradiography on brains from rats withdrawn from diazepam. As this method was well established in my laboratory the research assistant embarked on this aspect of the work first (see results in above section). The result of our investigations into the methodology for glutamate microdialysis was that a method involving a gradient system with fluorescence detection was highly preferable to the isocratic method which we had originally proposed. This necessitated purchasing additional equipment (Fluorescent detector and additional HPLC pump). The full monies for these were not available to us even if we did not purchase the autosampler as stated on the original grant. An application for this equipment was made to the Department and was successful although the equipment did not arrive until June 1998.
The final delay has been a result of our relocation to a new building which has resulted in the ceasing of animal work from July 1998 to date.

Despite these problems, some progress has been made. Dopamine measurements can be made in freely moving rats using HPLC with electrochemical detection. In line with other groups we have shown that acute diazepam treatment reduces extracellular

dopamine concentrations in the nucleus accumbens. This also appears to be the case after chronic treatment. Following treatment with diazepam (2.5 mg/kg), dopamine concentration in the accumbens were reduced by 49% one hour after treatment and had returned to control levels by 2 hours. As part of our general interest in the relationship between glutamate and dopamine transmission in the accumbens, we have also investigated the effects of the orally active AMPA antagonist GYKI 52466 upon extracellular dopamine concentrations in the accumbens and found dopamine levels to be unaffected.
Further studies are planned as our new facilities become available. These will also be conducted by Miss Allison whom I have now securred more long term funding for (four years) from the University of Strathclyde. This will enable her to complete her PhD and gain teaching experience.

Conditioned Place Preference Studies

As part of our more general interest in the reinforcing properties of addictive drugs we were interested in testing the hypothesis that glutamate receptors are involved in the reinforcing properties of diazepam. There are a number of reasons why this is of interest. Unlike many addictive drugs, benzodiazepines do not increase extracellular dopamine concentrations in the nucleus accumbens. In fact, we and others have demonstrated benzodiazepines reduce dopamine concentrations in the accumbens. Despite this, 6-hydroxydopamine lesions of the nucleus accumbens attenuate diazepam-induced conditioned place preference (Spyraki and Fibiger 1988, Psychopharmacology, 94, 133-137.) Taken together these data suggest additional neural systems may be important in regulating the outflow of information from the accumbens. Possibilities include the glutamatergic afferents from the amygdala and hippocampus. Interestingly, antagonism of AMPA/kainate receptors in the accumbens blocks amphetamine-induced place preference.

In our studies we have investigated the ability of the orally active AMPA/kainate receptor antagonist, GYKI 52466 to prevent diazpam-induced conditioned place preference. Using the place preference paradigm of Spyraki and Fibiger (1998), we found that diazepam could induce a significant place preference at doses of 2.5 and 5.0 mg.kg. Pretreatment with GYKI 52466 (4.8 mg/kg i.p) during the conditioning trials prevented the acquisition of diazepam-induced place preference. These data suggest that AMPA/kainate receptors are involved in the reinforcing properties of diazepam. Results from our microdialysis studies revealed that GYKI 52466 does not alter dopamine concentrations in the accumbens *per se*. Taken together these results suggest that glutamate receptors are important in the reinforcing properties of benzodiazepines. Furthermore, our findings add weight to the concept that the reinforcing properties of drugs of abuse cannot simply be attributable to activation of the mesoaccumbens dopamine system *per se* but that other neural systems which may impinge on the mesolimbic system are involved to confer these properties. We are continuing this work to explore further the neural substrates underlying diazepam reinforcement and the relationship between dopamine and glutamate transmission in these effects.

Results from this aspect of the research programme have been submitted to the journal **Neuroreport**. A copy of the manuscript is attached.

The neurochemistry of benzodiazepine withdrawal.

Investigators: Dr J. Pratt and Ms C. Allison, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow.
Contact person: Dr J Pratt

The overall objective of this research was to test the hypothesis that changes in glutamate transmission are important in benzodiazepine withdrawal. We have demonstrated that withdrawal from chronic diazapam treatment results in regionally selective increases in [^{3}H] AMPA receptor binding as determined by quantitative receptor autoradiography. Significant increases in binding occured in the CA2 field of the hippocampus and the frontal cortex. These data can account, at least in part, for the regional changes in activity of circuits recruited during chronic benzodiazepine treatment. We conclude that alterations in glutamate transmission are part of the neuroadaptive processes that occur during benzodiazepine dependence.

In microdialysis studies we have demonstrated that diazepam reduces extracellular dopamine concentrations in the nucleus accumbens. Thus benzodiazepines produce opposite effects to other addictive drugs upon dopamine transmission which have consistently been reported to increase accumbens dopamine concentration. This led us to investigate a role for glutamate receptors in the reinforcing properties of benzodiazepines. We have shown that the orally active AMPA/kainate receptor antagonist, GYKI 52466 blocks the acquisition of diazepam-induced conditioned place preference. Furthermore our microdialysis studies reveal that GYKI 52466 does not alter dopamine concentrations in the accumbens *per se*. Taken together these results suggest that glutamate receptors are important in the reinforcing properties of benzodiazepines. Future studies on the relationship between dopamine and glutamate transmission should reveal new insights into the neural mechanisms underlying the reinforcing properties of addictive drugs.

Submitted to Neuroreport

A role for AMPA/kainate receptors in diazepam-induced conditioned place preference

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Running title:
AMPA receptors and diazepam reinforcement

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ABSTRACT

In contrast to many other dependence producing drugs, the role of dopamine in the reinrorcing properties of benzodiazepines remains unclear. Activation of AMPA/kainate receptors are implicated in drug-induced reinforcement but a role for this receptor in benzodiazepine-induced reinforcement has not been examined. In the present study, diazepam (2.5 and 5.0 mg kg^{-1}, i.p.) produced a robust place preference. The orally active AMPA/kainate receptor antagonist GYKI 52466 inhibited the acquisition of place preference conditioning-induced by diazepam. These results suggest that glutamatergic pathways are an important component of the circuitry involved in the acquisition of a benzodiazepine induced place preference.

Key words: AMPA/kainate antagonist; diazepam, conditioned place preference; reinforcement; benzodiazepine, dopamine.

Introduction

The abuse liability of drugs is considered to be related to their positive reinforcing properties. These properties are well documented for the psychostimulant and opiate classes of drugs in self administration and conditioned place preference paradigms in various species [1,2]. However, despite their abuse potential, it is difficult to reliably demonstrate self-administration of the benzodiazepine tranquillisers [3]. This may be related to the interfering effects of the sedative and muscle relaxant properties of the drugs upon the animals ability to perform an operant task. In contrast, the reinforcing properties of benzodiazepines have been demonstrated in the conditioned place preference paradigm [4].

The neural circuitry and the neurotransmitters critical to the reinforcing properties of addictive drugs remains to be fully defined. Nevertheless, it is clear that the nucleus accumbens is central to this circuitry [1,5]. The convergence of many neural inputs to this region suggests that multiple brain areas and neurotransmitters are involved. The dopaminergic projection from the ventral tegmental area (VTA) has received most attention and there is strong evidence that the reinforcing properties of opiates and psychostimulants is dependent upon increased dopamine transmission in the projection areas of the VTA. Furthermore it has been suggested that a common neurochemical feature of all drugs of abuse is their ability to increase extracellular dopamine levels in the nucleus accumbens [6]. The benzodiazepines however differ from nicotine, opiates and amphetamine in that they reduce rather than increase dopamine concentrations in the nucleus accumbens[7]. Despite this, 6-hydroxydopamine lesions of the nucleus accumbens attenuated diazepam-induced conditioned place preference [8]. Taken together these data

suggest that additional neural systems may be important in conferring the reinforcing properties of benzodiazepines. This could be achieved through the integration of other inputs to the accumbens or through the involvement of different brain areas. In light of the close relationship between GABA and glutamate transmission in areas such as the cortex and hippocampus it is likely that glutamate systems may have an important role to play in the establishment of benzodiazepine induced conditioned place preference. Possibilities include the glutamate projections to the accumbens from areas including the amygdla and hippocampus [9]. Since there is a GABAergic output from the accumbens to the ventral pallidum it is possible that an alteration in the balance of excitatory and inhibitory transmission in the accumbens is important for the development of place preference induced by diazepam. In addition, antagonism of AMPA/kainate receptors in the accumbens blocks the acquisition of a place preference induced by amphetamine but not one induced by morphine[10]. The present study was aimed at determining whether the orally active AMPA/kainate antagonist GYKI 52466 could prevent diazepam induced conditioned place preference.

Materials and Methods

Groups of male Long-Evans hood rats were divided into the following treatment groups; diazepam (2.5 or 5.0 mg/kg i.p.) alone, GYKI 52466 (4.8 mg/kg i.p) alone, GYKI 52466 plus diazepam, and vehicle (1% Tween 20 in saline). GYKI 52466 or saline was administered 5min prior to diazepam or vehicle. The CPP method employed was

according to that used by Spyraki and Fibiger 1988[8]. Thus a three compartment apparatus was employed with guillotine doors separating the compartments. A small central compartment separated 2 larger compartments; one of which was painted grey and the other with black and white stripes. The floor of the latter compartment consisted of irregular perspex. The behaviour of rats was monitored using a video camera in an adjacent room. On the three days prior to drug administration and conditioning, rats were allowed to freely explore the apparatus for 15min with the doors open. The baseline preference was recorded on day three, with the chamber they spent the least time in considered the non-preferred side. Rats were then subjected to four conditioning trials. GYKI 52466 (4.8 mg/kg i.p.) or vehicle was administered on days 1,3,5 and 7 five min prior to diazepam (2.5 mg/kg i.p.) or vehicle, and, 10min later rats were confined to the initially least preferred side for 30 mins. On days 2, 4, 6 and 8 all groups were injected with vehicle and confined to the initially preferred side. Rats were tested for place preference on day 10 in which the animals were allowed to freely explore the apparatus for 15 min and the time spent in each side recorded. The difference in time spent in the drug-paired compartment between the final test trial (day 10) and the last day of the preconditioning period represents a measure of place conditioning, with a positive value in favour of the drug compartment reflecting appetitive properties of the drug [4]. Data was analysed by MANOVA followed where appropriate by the Newman Keuls multiple range test.

Results

The effects of two doses of diazepam upon place preference conditioning were investigated in order to determine the most suitable dose for the subsequent antagonist study. The groups of rats injected with diazepam showed a significant shift in preference towards the side that had been associated with the drug ($P< 0.05$) (table 1). There was no such shift in preference in the vehicle treated control group. Whist there was no significant difference between the two diazepam treatment groups, the effect of diazepam was qualitatively more apparent at a dose of 2.5mg kg^{-1} compared to 5 mg kg^{-1} in line with previous studies[4].

Pretreatment with the AMPA/kainate antagonist GYKI 52466 significantly antagonised diazepam-induced conditioned place preference (Fig 1). Thus there was a significant between group effect [$F(4,35) = 4.14$, $P < 0.005$] . Post hoc analysis revealed that the vehicle-diazepam group was significantly different from the GYKI 52466-diazepam group ($P< 0.05$). Similarly, the vehicle-diazepam group and the vehicle-GYKI 52466 groups were significantly different from the vehicle control group ($P< 0.05$).

Discussion

Diazepam at both doses tested produced a conditioned place preference. The apparent lack of a clear dose dependence of this effect is in keeping with previous findings[4] . One explanation for the lack of ability of the 5mg/kg dose of diazepam to produce an increased place preference as compared to the smaller dose of 2.5 mg/kg could be

attributable to the potentially confounding effects of sedation at the larger dose. Whilst the present study did not monitor locomotor activity in the apparatus, previous studies have discussed the importance of drug-induced changes in locomotor activity as a factor in determining whether drugs can produce conditioned place preference [2].

The present study demonstrates that the AMPA/kainate antagonist, GYKI 52466 can prevent the acquisition of diazepam (2.5 mg/kg)-induced place preference suggesting a role for these receptors in diazepam reinforcement. A role for AMPA/kainate receptors in the acquisition of place preference conditioning induced by amphetamine but not one induced by morphine has been reported previously[10]. The nucleus accumbens appeared to be an important locus in mediating the effect of DNQX upon amphetamine conditioning[10]. AMPA/kainate antagonists, including GYKI 52466 as well as NMDA receptor antagonists have also been reported to block the expression of conditioned place preference induced by morphine and amphetamine[10,11].

Taken together these data suggest that AMPA/kainate receptors are involved in the primary reward stimulation of diazepam and amphetamine (acquisition of place preference) but not morphine. This is supported by the findings that different neural substrates are involved in the activation of reward circuitry evoked by psychostimulants and opiates[1,12]. Thus for amphetamine and cocaine the nucleus accumbens and prefrontal cortex are important whereas several structures including the ventral tegmental area, nucleus accumbens, hippocampus and hypothalamus have been implicated in the rewarding properties of opiates[1,12].

The neural substrates critical to the reinforcing properties of benzodiazepines remain unclear. Previous studies have demonstrated that 6-hydroxydopamine lesions of the nucleus accumbens prevented diazepam-induced place preference whereas lesions of the noradrenergic system were without effect[8]. This would be in keeping with data from other drugs of abuse implicating the nucleus accumbens in reinforcement. However the paradox is that in contrast to other drugs of abuse, benzodiazepines reduce rather than increase extracellular concentrations of dopamine in the accumbens [6,7]. Similarly diazepam has been shown to block morphine-induced place preference and morphine associated increases in dopamine turnover in the limbic forebrain[13]. These data suggest that while the accumbens may be involved in the reinforcing properties of diazepam this may be independent of increased dopamine release. Consideration of alternative neurochemical mechanisms is therefore required. Based upon the present results, one possibility would be that benzodiazepines increase glutamate transmission within the nucleus accumbens. Conceivably this glutamatergic input could arise from structures such as the amygdala and hippocampus. The exact mechanism through which benzodiazepines could enhance glutamate transmission remain unclear. In the hippocampus for example, the location of GABAergic interneurones would suggest reductions in glutamate afferent activity to the acccumbens. However, there is no evidence that the benzodiazepine agonist diazepam decreases the extracellular levels of glutamate despite benzodiazepine receptor inverse agonists increasing glutamate efflux in the prefrontal cortex[14]. Based upon the present knowledge it is therefore difficult to envisage how benzodiazepines could directly increase the activity of glutamate afferents to the accumbens from areas such as the amygdala and hippocampus. It remains

possible that benzodiazepines indirectly modify glutamatergic systems through other neuronal systems.

Some other aspects of the place preference paradigm should be considered in interpreting the present data. For example we used an unbalanced paradigm in which the place preference produced by diazepam could be interpreted as reduction of an aversion to an unpleasant environment. This seems possible in light of the anxiolytic properties of diazepam. However, the possibility that diazepam-induced conditioned place preference is the result of the anxiolytic rather than the rewarding effects of the drug has been ruled out in previous studies which employed the same paradigm[4].

An alternative explanation for the ability of GYKI 52466 to prevent the place preference conditioning evoked by diazepam is that it produces an opposite effect to that of diazepam and that the block of the effects of diazepam is simply the result of opposing effects. However, this possibility is ruled out by the finding that GYKI 52466 is not aversive in our paradigm.

An interesting finding of the present study was that GYKI 52466 produced a modest place preference when administered alone. These findings are in keeping with a recent study which demonstrated that inhibition of glutamate release by riluzole could induce a conditioned place preference. Moreover, this compound could block place preference induced by amphetamine and morphine[15]. However, other studies have not shown a reinforcing effect with GYKI 52466[11].

In summary, the present findings suggest a role for AMPA/kainate receptors in the acquisition of the reinforcing properties of diazepam and implicate glutamate pathways in the reinforcing properties of benzodiazepines.

Conclusions

Diazepam produced a conditioned place preference, the acquisition of which could be blocked by the orally active AMPA/kainate antagonist GYKI 52466. These results suggest that enhanced glutamate transmission may be important in the acquistion of the reinforcing effects of diazepam and support a role for AMPA/kainate receptors in this effect. Furthermore, our findings add weight to the concept that the reinforcing properties of drugs of abuse cannot simply be attributable to activation of the mesoaccumbens dopamine system *per se*. Rather recruitment of other neural systems which impinge on the mesolimbic dopamine system may act in concert to confer the appetitive properties of benzodiazepines. Finally, consideration of the distribution of the receptor proteins through which drugs of abuse exert their primary site of action should be accounted for in models predicting the neural substrates critical to drug reinforcement.

Acknowledgements

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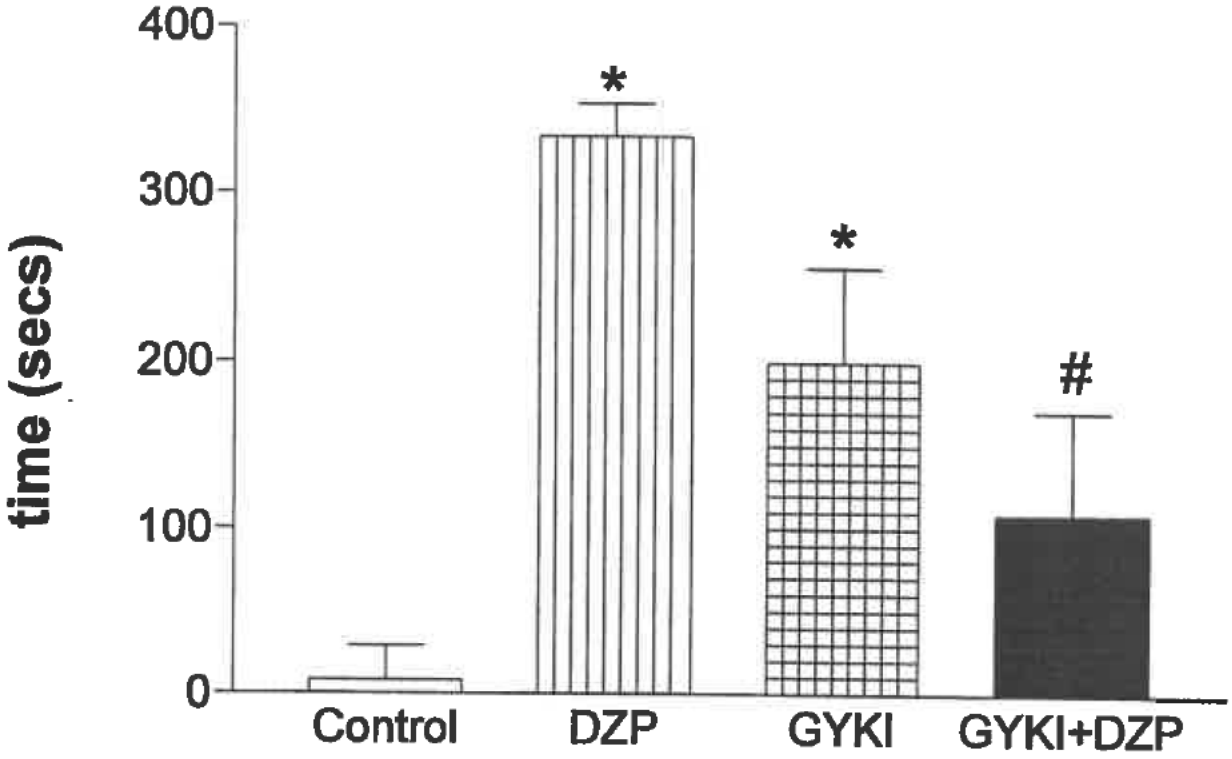
Table 1. Conditioned place preference induced by diazepam.

Group	Pre-Conditioning	Post-Conditioning
Vehicle	149±19	157±22
Diazepam ($2.5\ mg\ kg^{-1}$)	196±20	530±20*
Diazepam ($5.0\ mg\ kg^{-1}$)	137±17	369±25*

Values represent mean (±SEM) time (s) spent in the initially least preferred environment before and after conditioning. * $P < 0.05$

Fig 1. Effect of GYKI 52466 (GYKI) on diazepam (DZP)-induced place preference. Data represent means (±SEM) of the differences in time (s) spent in the drug-paired side between pre- and postconditioning test session. * $P < 0.05$ compared to vehicle control, # $P < 0.05$ compared to vehicle-diazepam group.


400
300
200
100
0
time (secs)
*
*
#
Control
DZP
GYKI
GYKI+DZP

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Regional Alterations in [^{3}H]AMPA binding in rat brain after chronic diazepam treatment

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The neuronal mechanisms underlying dependence on benzodiazepines remain unclear. Changes at the level of the $GABA_A$ receptor are inadequate as an explanation for the changes in neural activity in circuits recruited during chronic benzodiazepine treatment (Pratt et al. 1998). The present study focuses on the hypothesis that the chronic enhancement of GABAergic inhibition by diazepam causes compensatory increases in excitatory glutamatergic mechanisms. The aim of these experiments was determine if chronic diazepam treatment leads to regionally specific changes in AMPA receptor binding.

Experiments were carried out on male Hooded Long Evans rats (200-300g; n = 9-10 per group). Rats were randomly assigned to 1 of 3 treatment groups, either daily injection of vehicle for 28 days, daily injection of vehicle for 14 days followed by daily injection of diazepam (5mg Kg^{-1} i.p.) for 14 days or daily injection of diazepam (5mg Kg^{-1} i.p.) for 28 days. Twenty four hours following the last injection, rats were killed and the brains removed and frozen in isopentane before being stored at -70°C. 20µm brain sections were cut at 8-9 selected levels and prepared for [^{3}H]AMPA receptor autoradiography following a protocol modified from Dev & Morris (1994). Sections were incubated with 10nM [^{3}H]AMPA (specific activity = 40.6 Ci/mmol), using 10µM quisqualate to define nonspecific binding. Resultant autoradiograms were analysed using computer based densitometry (MCID). Differences in the levels of specific [^{3}H]AMPA binding in each brain area were analysed by one-way ANOVA, followed by the Student Newman-Keuls multiple range test if appropriate.

Following 28 days treatment with diazepam (5mg Kg^{-1} i.p.) there was an increase in specific [^{3}H]AMPA binding in the frontal cortex and in the CA2 field of the hippocampus relative to control. 14 days treatment with diazepam (5mg Kg^{-1} i.p.) also produced an increase in specific [^{3}H]AMPA binding in the CA2 region of the hippocampus relative to control (Table 1).

These data provide preliminary evidence which supports the hypothesis of enhanced glutamatergic mechanisms following chronic benzodiazepine treatment.

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Brain area	Control (Vehicle treated)	14 days 5mg Kg^{-1} Diazepam	28 days 5mg Kg^{-1} Diazepam
Frontal Cortex	1883 ± 313.9	2396 ± 258.5	3024 ± 223.0*
Cingulate Cortex	1628 ± 296.0	1716 ± 299.5	1571 ± 341.4
CA1 Field Hippocampus	3159 ± 255.4	3588 ± 527.2	3311 ± 490.2
CA2 Field Hippocampus	1071 ± 169.9	2864 ± 573.7*	2608 ± 444.3*

Table 1. [^{3}H]AMPA specific binding (nCi/g) in rat brain. Data expressed as mean ± sem (n = 4-9 per group). * $p<0.05$ vs vehicle treated animals.