QUASI-MORPHINE ABSTINENCE BEHAVIOUR GABA-ERGIC MECHANISMS AND THEIR LOCALIZATION

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 23 SEPTEMBER 1981 DES NAMIDDAGS
TE 3.45 UUR

DOOR

Jan Willem van der Laan

geboren te Groningen

krips repro meppel

PROMOTOR: Prof. Dr. J. Bruinvels

CO-REFERENTEN: Prof. Dr. J.F. Koster
Prof. Dr. J. Moll

Het onderzoek dat leidde tot dit proefschrift werd verricht op de afdeling Farmacologie van de Erasmus Universiteit Rotterdam (hoofd: Prof. Dr. I.L. Bonta).
Het werd gesteund door de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO (project 13-47-11), die wordt gesubsidieerd door de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (Z.W.O.)

Omslag-ontwerp: Studio Myosotis, Baarn.

Voor Nieske
Voor Els, Rik en Janneke
Voor mijn ouders

Met dank aan de Here, die mij de mogelijkheden gaf dit werk te volbrengen.

Bij het gereedkomen van dit proefschrift wil ik graag iedereen bedanken die op enigerlei wijze geholpen heeft bij de uitvoering van het onderzoek en bij het tot stand komen van dit boekje.

Om te beginnen wil ik mijn promotor, Dr. J. Bruinvels, bedanken. Jacques, door jouw positieve benadering in het onderzoek heb je mij erg gestimuleerd en van jouw meestal niet malse kritiek, vooral bij het schrijven, heb ik erg veel geleerd.

De directie van het Rijks Instituut voor de Volksgezondheid dank ik voor de mogelijkheid om een deel van mijn tijd vrij te maken voor het schrijven van mijn proefschrift.

Mike Parnham wil ik bedanken voor het corrigeren van mijn moeizame Engels, iets wat vooral op het laatst in no-time moest gebeuren. Els Hillen bedank ik voor de uitstekende uitvoering van het tikwerk om dit proefschrift persklaar te maken.

Daarnaast bedank ik Kees Aalbers, Aalt Bast, Peter Bragt, Thijs de Boer, Magda Busscher-Lauw, Lex Cools, Wistaria Cairo-Rawlins, Diny Franchimont, Feddo Hillen, Fons Jacobs, Ton van de Kuil, Jos Lamers, Peter Moleman, Jan Noordhoek, Willem Oudejans, Loeky van de Poel-Heisterkamp, dhr. Schoelitz, Yvonne Tjoa-Lie, Kees van Valkenburg, Marijke Verhulst, Gradus Weick, Paul van Wel, Amarentia Wijling, Freek Zijlstra, dhr. Schuhmacher en zijn medewerkers van de CRW, John Bovenlander van de ASW en de medewerkers van de AVD voor de ondervonden medewerking en verder ook alle niet met name genoemde (ex)medewerkers van de afdeling Farmacologie voor het onderhouden van een prettige werksfeer.

Tenslotte, Nieske, jou wil ik bedanken voor de moeite die jij je hebt getroost om mij te laten zien dat de wereld uit meer dan Farmacologie bestaat en voor het geduld wat je vooral de laatste jaren hebt betoond.

Contents

Abbreviations used:

Ac	-	central amygdala
AOAA	-	aminooxyacetic acid
BMI	-	bicuculline-methiodide
Cm-Pf	-	centre median-parafascicularis area
DMV	-	2,2-dimethylvalerate
DPA	-	di-n-propylacetate
GABA	-	4-aminobutyrate
GABA-T	-	4-aminobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.19)
GAD	-	L-glutamate 1-carboxylyase (EC 4.1.1.15)
Glu	-	glutamate
5HTP	-	5-hydroxytryptophan
K	-	Michaelis constant under saturated conditions
K_m	-	apparent Michaelis constant under non-saturated conditions
LC	-	locus coeruleus
MEC	-	2-methyl,2-ethylcaproate
NA	-	noradrenaline
2-OG	-	2-oxoglutarate
3OHDPA	-	3-hydroxy,2-propylvalerate
4OHDPA	-	4-hydroxy,2-propylvalerate
5OHDPA	-	5-hydroxy,2-propylvalerate
PLP	-	pyridoxal-5'phosphate
SA	-	succinate
SSA	-	succinate-semialdehyde
SSA-DH	-	succinate-semialdehyde: $NAD(P)^+$oxidoreductase (EC 1.2.1.16)
TRH	-	thyrotropin-releasing hormone
V	-	maximum velocity under saturated conditions
V_{max}	-	apparent maximum velocity at a fixed non-saturating concentration of a single substrate

CHAPTER I

GABA-ERGIC MECHANISMS AND MORPHINE ABSTINENCE BEHAVIOUR: A SURVEY OF THE LITERATURE

1.1. INTRODUCTION

1.2. GABA-ERGIC MECHANISMS

1.2.1. Metabolism and distribution

1.2.2. Shunt enzymes

1.2.3. Synaptic events

1.2.4. Metabolic compartmentation

1.2.5. GABA and convulsions

1.2.6. Di-n-propylacetate

1.2.7. GABA and behaviour

1.3. MORPHINE ABSTINENCE BEHAVIOUR

1.3.1. Morphine abstinence behaviour, definitions and description

1.3.2. Quasi-morphine abstinence behaviour

1.3.3. Localization of sites involved in abstinence behaviour

1.3.4. Morphine actions on the GABA system

1.4. DPA-INDUCED QUASI-MORPHINE ABSTINENCE BEHAVIOUR

1.4.1. Methods

1.4.2. The role of GABA

1.4.3. Aim of this thesis

1.1. INTRODUCTION

Since the discovery of the non-protein amino acid, gamma-aminobutyric acid (GABA), in mammalian brain (Roberts and Frankel, 1950; Udenfriend, 1950; Awapara et al., 1950), evidence has been gathered indicating that this compound has a function as an inhibitory neurotransmitter (for a review see Krnjevic, 1970). Subsequent to its discovery in the brain GABA has also been identified in other tissues, although in most cases only traces could be found (Zachman et al., 1966; Whelan et al., 1969; Gerber and Hare, 1980).

Recently, it has been suggested that GABA may fulfil a role in morphine abstinence behaviour. A syndrome resembling morphine abstinence behaviour can be induced in rats using di-n-propylacetate (DPA), a compound presumably acting through GABA. The role for GABA in this behaviour was suggested by its suppression by the GABA antagonists picrotoxin and bicuculline (De Boer et al., 1977, 1980). The results presented in this thesis provide further evidence for a role of GABA in this behaviour induced by DPA. In addition, the involvement of different brain areas in DPA-induced behaviour has been studied. The implications of these findings for the involvement of GABA in morphine abstinence behaviour will be discussed at the end of the thesis. In this first chapter, therefore, a short review will be given on GABA, morphine abstinence behaviour and the interactions between GABA and morphine.

In the first part of this chapter the recent literature concerning GABA will be reviewed with the emphasis on mechanisms regulating GABA-ergic activity. The metabolism of GABA, the enzymes involved in this metabolism and the synaptic events related to GABA will be discussed. Furthermore, a short description will be given of the metabolic compartmentation of GABA and related substances. Since DPA is an anticonvulsant drug, the role of GABA in convulsions and the hitherto assumed mechanism of action of DPA will be discussed. In order to compare the behavioural effects of DPA with the effects of other GABA-ergic compounds an overview will be given of the effects of these compounds on the spontaneous behaviour of rats and mice.

In the second part of the chapter the following subjects will be discussed: the terms involved in the study of morphine abstinence behaviour; quasi-morphine abstinence behaviour and its implications for true morphine abstinence; the brain areas responsible for morphine abstinence; the interaction between morphine and GABA.

Finally, a description is given of what was known about DPA-induced behaviour before the research described in this thesis was initiated,

together with an explanation of the way in which we have extended this research by the present experiments.

1.2. GABA-ERGIC MECHANISMS

1.2.1. Metabolism and distribution

GABA is synthesized in the brain mainly by decarboxylation of L-glutamate by the enzyme glutamate decarboxylase (GAD; L-glutamate. 1-carboxy-lyase; EC.4.1.1.15). The major pathway of GABA-degradation is its transamination by GABA-transaminase (GABA-T; 4-aminobutyrate: 2-oxoglutarate aminotransferase; EC.2.6.1.19) and subsequent oxidation to succinate by succinic semialdehyde dehydrogenase (SSA-DH; succinic semi-aldehyde: NAD^+ oxido-reductase; EC. 1.2.1.16).

Since glutamate can be formed from 2-oxoglutarate, an intermediate of the Krebs cycle, the conversion of 2-oxoglutarate via glutamate and GABA to succinate is referred to as a bypass of the Krebs cycle and is called the GABA shunt, as shown in Fig. 1.2.1.

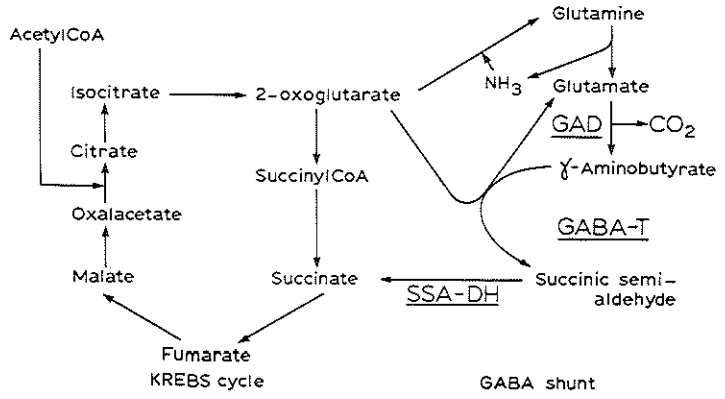

FIG. 1.2.1. The relation between KREBS cycle and GABA shunt. The enzymes belonging to the GABA shunt are underlined.

The distribution of GABA in rat brain has been studied by many authors (e.g. Balcom et al., 1975; Tappaz et al., 1977; Van der Heyden and Korf, 1978). Van der Heyden et al. (1979 b) have measured the GABA content of approx 70 nuclei. GABA is present in nearly all areas of the brain. High levels of

GABA are present in the hypothalamus, substantia nigra and globus pallidus.

The GABA concentration in nerve terminals has been estimated to be 60-140 mM in the cat (Fonnum and Walberg, 1973 a,b; Fonnum et al., 1974) and 10-20 mM in the rat (Storm-Mathisen, 1976). Whether or not this GABA is stored in synaptic vesicles, like the storage of catecholamines (Douglas, 1968), is still a matter of dispute. The association of GABA with vesicular structures, a quantity accounting for only 10-20% of total brain GABA (Kuriyama et al., 1968 a,b; Kuriyama, 1976; Zisappel and Zurgil, 1978), may be loose and it probably represents unstable compartments (Mangan and Whittaker, 1966).

In conclusion, GABA is present in nearly all areas of the brain, with high concentrations in the hypothalamus, substantia nigra and globus pallidus. The concentration of GABA in nerve endings may be in the order of 10-50 mM.

1.2.2. Shunt enzymes

Each enzyme involved in the synthesis and degradation of GABA has its own cofactor requirements and kinetic profile, the modification of which can lead to changes in the ultimate concentration of GABA.

The enzyme GAD, responsible for the synthesis of GABA, requires pyridoxal-5'phosphate (PLP) as a cofactor. The enzyme has been purified from mouse, rat and human brain (Wu et al., 1973; Maitre et al., 1978 b; Blindermann et al., 1978) and is located mainly in nerve endings (Salganicoff and DeRobertis, 1963, 1965; Balasz et al., 1966; Wood et al., 1976).

GAD activity can be inhibited by some Krebs cycle intermediates and GABA (Gerig and Kwock, 1979; Wu and Roberts, 1974; Blindermann et al., 1978). The latter effect may be important for feedback regulation of the GABA level in nerve terminals (Bayon et al., 1978; Nitsch, 1980).

The activity of GAD can be influenced too by changing the degree of saturation of the apoenzyme for the coenzyme PLP, since the *in vivo* saturation of GAD by PLP is only 24-47% (Miller et al., 1977; Nitsch, 1980). Pharmacological manipulation of the synthesis of GABA *in vivo*, based on competition with PLP, results in convulsions or in a decrease in the convulsive threshold (see for a review: Péréz de la Mora et al., 1973).

GABA-T, the first enzyme in the degradation of GABA, has been purified from mouse, rat, rabbit, pig, bovine and human brain (Schousboe et al., 1973; Sytinski and Vasilijev, 1969; Waksman and Roberts, 1965; Maitre et al., 1975; John and Fowler, 1976; Bloch-Tardy et al., 1974; Kobayashi et al., 1977; Maitre et al., 1978 a). Waksman and Bloch (1968) have purified and

separated several isozymes of GABA-T, which seem to be located in different subcellular organelles. GABA-T from non-synaptosomal mitochondria has a much higher activity and a higher affinity for GABA (Km for GABA is 4-6.5 mM) than the GABA-T located in synaptosomal mitochondria (Km for GABA is 30-50 mM) (Ngo and Tunnicliff, 1978). The weaker affinity of synaptosomal GABA-T for GABA and the lower activity of this enzyme favour a role of GABA-T in the regulation of the high presynaptic GABA level (see section 1.2.1).

It has been suggested that the low activity of GABA-T in synaptosomes can be attributed to contamination of this fraction with free cytoplasmic mitochondria (Balász et al., 1966; Van den Berg et al., 1975; Hertz, 1979). However, electronmicroscopic studies and measurements of marker enzymes indicate that the low GABA-T activity detected in these fractions cannot be due to such a contamination (Salganicoff and DeRobertis, 1965; Van Kempen et al., 1965; Waksman et al., 1968; Walsh and Clark, 1976). Recently, the localization of GABA-T in nerve endings has been unequivocally established using immunohistochemical methods (Barber and Saito, 1976; Chan-Palay et al., 1979).

A high ionic strength has been shown to decrease the activity of GABA-T (De Boer and Bruinvels, 1977 a). In addition, the purified enzyme is susceptible to inhibition by a large number of metal ions, glutamate and some other naturally occurring substances (Schousboe et al., 1974). Interference from carbohydrate metabolism, e.g. hypoglycemia, will also affect GABA-T activity, through alterations in the level of 2-oxoglutarate (Otsuki et al., 1968; Dravid and Jilek, 1965).

SSA-DH, the second enzyme of the GABA degradation, appears to be more specific for the GABA shunt than GABA-T (Embree and Albers, 1964; Albers and Koval, 1961). The enzyme has been purified from monkey, human and rat brain (Albers and Koval, 1961; Embree and Albers, 1964; Kammeraat and Veldstra, 1967; Cash et al., 1975, 1977).

Just like GABA-T, SSA-DH activity seems to be present in mitochondria of both synaptosomal and non-synaptosomal origin (Salganicoff and DeRobertis, 1965; Sims and Davis, 1973). The distribution of the enzyme in the brain parallels the distribution of GABA-T too (Miller and Pitts, 1967; Sheridan et al., 1967). The activity of SSA-DH is always higher (1.5-5 times) than that of GABA-T (Miller and Pitts, 1967; Cash et al., 1975; De Boer and Bruinvels, 1977 a). This higher activity of SSA-DH (as compared to GABA-T), together with its high affinity for SSA, indicate that GABA-T and not SSA-DH is rate-limiting in the degradation of GABA. However, changes in the

concentration of cations (De Boer and Bruinvels, 1977 b; De Boer et al., 1979) or in the availability of the cofactor NAD^+, e.g. during hypoxia (Gubler et al., 1974), may diminish the activity of SSA-DH in such a manner that it may become rate limiting.

In conclusion, the GABA synthesizing enzyme GAD is located almost exclusively in nerve terminals and may be regulated by PLP availability and by the intraterminal GABA concentration. GABA degradation occurs mainly, but not exclusively, in the non-nerve terminal compartment. It is suggested that different isozymes of GABA-T are located in nerve terminals and in glial cells. Cation concentration as well as ionic strength may regulate the activity of the enzymes involved in the GABA degradation.

1.2.3. Synaptic events related to GABA

In accordance with its role as a putative neurotransmitter, GABA is released into the synaptic cleft, interacts with specific receptors and is inactivated by uptake into the neurons or the glial cells surrounding the synaptic cleft.

Release of GABA has been demonstrated to occur after electrical stimulation or by depolarizing potassium concentrations both *in vitro* (Katz et al., 1969; Srinivasan et al., 1969) and *in vivo* (Obata and Takeda, 1969; Van der Heyden et al., 1979 a). It has been postulated by Tapia et al. (1975) that GABA is continuously released from the synaptosomal cytoplasm of inhibitory nerve endings with a rate depending on the activity of GAD. According to these authors it is still possible that GABA may also be released from a storage site such as synaptic vesicles, when the GABA neuron is depolarized by a stimulus. The existence of a feedback control of the release of GABA via presynaptic autoreceptors has been indicated by both *in vitro* and *in vivo* experiments (Mitchell and Martin, 1978; Van der Heyden et al., 1980; Brennan et al., 1981).

Binding of GABA to subcellular brain particles has been shown to be either sodium-dependent or -independent (Sano and Roberts, 1961; Zukin et al., 1974; Enna and Snyder, 1975). The sodium-independent binding of GABA represents binding to postsynaptic receptor sites since it could be prevented by bicuculline-methiodide (BMI) (for a review see De Feudis, 1978). The existence of more than one single type of GABA receptor has been suggested (Nistri and Constanti, 1979).

Regulation of the responsiveness of GABA receptors is possible by

desensitization of receptor affinity after chronically enhanced GABA-ergic stimulation (Enna et al., 1980) or by development of supersensitivity of the receptor as occurs after degeneration of GABA-ergic cells (Kuriyama et al., 1980). The sensitivity of the GABA receptor may also be altered by interaction with "GABA modulin", a low molecular weight protein, which appears to interact specifically with the GABA receptor (Toffano et al., 1978, 1980). It is not inconceivable that both the supersensitivity and subsensitivity phenomena occur via alteration in GABA modulin receptor interaction.

Uptake of GABA into the neuronal cell bodies or into the glial cells surrounding the synaptic cleft and not degradation is responsible for the termination of GABA-ergic transmission, since degradation of GABA requires the presence of Krebs cycle enzymes, which are located only in mitochondria (Schousboe, 1978; Iversen, 1971; Ryan and Roskoski, 1977; see review by Iversen and Kelly, 1975). Nerve terminals are also capable of taking up GABA (Fagg and Lane, 1979). The uptake systems for GABA are related to sodium-dependent GABA binding and it has been described that the GABA uptake depends on cationic fluxes across the membrane (Levi and Raiteri, 1978).

In conclusion, GABA-mediated neurotransmission can be regulated at the level of receptor interaction through modulation of the receptor affinity or at the level of release through a feedback system. The uptake may be regulated through alterations in ionic transport.

1.2.4. Metabolic compartmentation

The concept of metabolic compartmentation has been accepted for many years and refers to the presence in tissue of more than one metabolic pool. By definition, each pool of a certain compound has its own metabolic rate, which distinguishes the pool or compartment from another of the same compound. Such compartments are not in rapid equilibrium with each other; if they were, they would be indistinguishable from each other (Baxter, 1976).

There is much evidence suggesting that the glutamate in brain tissue is compartmentalized (for reviews see Balász and Cremer, 1972; Berl et al., 1975). The first indications of the existence of different glutamate pools were given in 1953 (Braganca et al., 1953) and in 1958 (Roberts et al., 1958). The latter authors studied glutamate metabolism in mouse brain after intraventricular administration of labelled GABA and they found that the specific activity of glutamine was higher than that of its precursor. glutamate. Brain glutamate metabolism has been suggested to consist of two

pools (Garfinkel, 1966; Van den Berg and Garfinkel, 1971). Other authors have proposed the existence of three compartments consisting of a small glutamate compartment in glial cells, a second small compartment composed of nerve terminals and a large compartment located in nerve cell bodies and dendrites (Balász et al., 1972 b). Several independent tricarboxylic acid cycles have also been identified in brain tissue and have been related to the different glutamate pools (Garfinkel, 1966). Both GABA synthesis and degradation are each divided over more than one compartment (Balász et al., 1972 a; Van den Berg et al., 1975). One has to bear in mind, however, that different metabolic compartments are not necessarily equivalent to morphologically different cell types. It is not inconceivable that, superimposed upon these morphological defined cell types, anatomically distinct brain divisions exist which represent separate metabolic compartments, independent of the number of cell types present.

Recently, Hertz (1979) has proposed four morphologically distinct compartments (Fig. 1.2.4), three of which include a complete Krebs cycle.

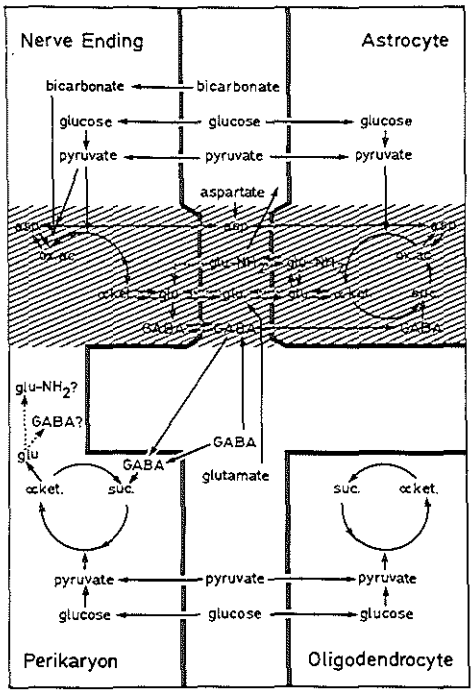

FIG. 1.2.4. Schematic representation of brain metabolism at the cellular level. To the left is shown a neuron with its nerve endings in the upper half and its non-synaptic parts (exemplified by the perikaryon) in the lower half. To the right is indicated an astrocyte in the upper half and remaining cell types, exemplified by an oligodendrocyte, in the lower half. The latter cell is not assumed to take part in any direct metabolic interaction with neurons or astrocytes. In between the cells is shown the extracellular space. From Hertz (1979).

The author suggested that GABA synthesis is located only in nerve endings, while GABA degradation takes place mainly in astroglia and neuronal perikarya. Therefore, a flux of GABA from nerve terminals to glial cells has to be present. Glutamine is transported in the opposite direction and may then act as the precursor of GABA in nerve endings (Tapia and Gonzalez, 1978; Reubi et al., 1978). Although the model fails in that degradation of GABA in nerve endings is not included (see section 1.2.2), it clearly demonstrates the complexity of GABA metabolism in the brain and the importance of the concept of metabolic compartmentation.

In conclusion, glutamate- and GABA metabolism is divided over several metabolic compartments. When considering the pharmacology of GABA, this compartmentation must be taken into account.

1.2.5. GABA and convulsions

GABA has been suggested to be implicated in many neuropathological and psychopathological states, e.g. schizophrenia (Roberts, 1972; Van Kammen, 1977), Huntington's chorea (Perry et al., 1973; Bird et al., 1973), Parkinson's disease (Bernheimer and Horniekiewicz, 1962; McGreer et al., 1971) and tardive dyskinesia (Gibson, 1977).

Since some work presented in this thesis deals with the mechanisms of action of the anticonvulsant drug di-n-propylacetate (DPA, Depakine^{R}), the possible relationship between GABA and convulsions will be discussed.

Before a physiological role for GABA as an inhibitory neurotransmitter was established, it had already been shown that GABA has anticonvulsive properties (Hayashi, 1959). A decreased availability of PLP, probably leading to a lower activity of GAD (see section 1.2.2), was suggested to be the cause of seizures occurring in children with a dietary vitamin B6 -deficiency (Holtz and Palm, 1964). This relationship between GABA and convulsions can be summarized as follows: a decrease in the level of GABA-ergic transmission parallels a decrease in the convulsive threshold and makes the animals more susceptible to convulsant agents. An increase in GABA-ergic transmission is associated with an increase in the convulsive threshold and protects the animals against convulsion-evoking treatments. Reviews on this topic have frequently been published (Meldrum, 1975; Tower, 1976; Meldrum, 1978; Roberts, 1980).
An anti-convulsive effect through an increase in GABA-ergic transmission can be achieved by administration of a GABA agonist (muscimol; Anlezark et al., 1978), by inhibition of GABA reuptake (L-2,4-diaminobutyric

acid; Taberner and Roberts, 1978; Horton et al., 1979), or by inhibition of GABA-transaminase (ethanolamine-O-sulphate; Anlezark et al., 1976; aminooxyacetic acid; Wallach, 1961; Kuriyama et al., 1966). Recently, reviews have been published on the inhibitors of GABA-T and their anticonvulsive action (Metcalf, 1979; Seiler and Sarhan,1980).

Although these reports suggest a simple relationship between GABA concentration in brain and the susceptibility to seizures, the relation appears to be more complex. Some compounds,e.g. hydrazides, which enhance GABA levels by inhibition of GABA-T, can, despite of enhancing GABA concentrations, induce convulsions. This is related to concomitant inhibition of GAD, and Wood and Peesker (1973) have postulated a mathematical relationship in which the excitability of the brain is determined not only by the GABA concentration but also by the GAD activity (see also Wood and Peesker, 1974, 1975; Wood, 1975). Other authors have also found that the level of synaptosomal GABA, which decreases primarily after GAD inhibition, is likely to be more important in determining excitability than total brain GABA (Abe and Matsuda, 1976, 1977; Matsuda et al., 1978, 1979; Wood et al., 1979, 1980). The specific involvement of synaptosomal GABA in the determination of brain excitability is further indicated by the finding that the onset and the peak of the anticonvulsive activity of the GABA-T inhibitor gamma-vinyl GABA (GVG) parallelled the time course for the (limited) increase in GABA in nerve endings induced by this compound and not that of the (massive) increase of GABA in the other compartments (Gale and Iadarola, 1980).

In conclusion, the increase in GABA concentration in the brain is probably involved in the anticonvulsive action of various compounds. The increased level of GABA in nerve terminals rather than in glial cells and other organelles seems to be important for this anticonvulsive action.

1.2.6. Di-n-propylacetate

Di-n-propylacetate (DPA) is an effective anticonvulsant which appears to act by raising GABA levels, since the time courses of its protection against convulsions and of its enhancement of the GABA level are similar both in rats and mice (Godin et al., 1969; Simler et al., 1973; Cieselski et al., 1975; Wood et al., 1977; Lust et al., 1978; LaColle et al., 1978). Fig. 1.2.6 represents the time course of the increase in GABA level in the brain after oral administration of 400 mg/kg DPA to rats. Regional studies, carried out in mice and rats (Simler et al., 1978; Iadarola et al., 1979), show that

the administration of DPA causes increased GABA levels of markedly varying size in different brain areas.

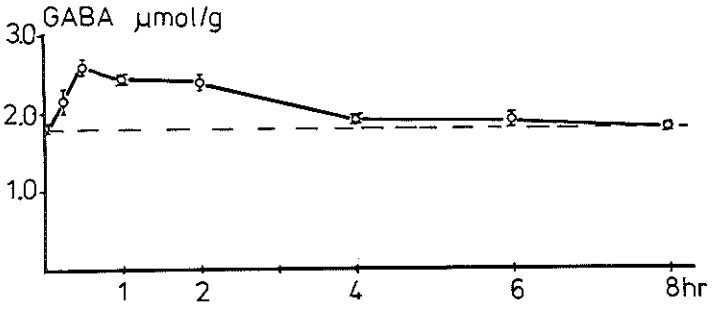

FIG. 1.2.6. Time course of the increase in GABA concentration in whole rat brain after oral administration of 400 mg/kg DPA. From Kukino and Deguchi (1977).

With respect to the effects of DPA on GABA-mediated neurotransmission, it has been shown that DPA augments GABA-ergic inhibition *in vitro* (Hayashi and Negishi, 1979; MacDonald and Bergey, 1979). Microiontophoretic application of DPA potentiates GABA-ergic inhibition *in vivo* too (Schmutz et al., 1979; Gent and Phillips, 1980) although, using the same method, enhancement of the excitability evoked by glutamate has also been reported (Blume et al., 1979). Intraperitoneal injection of DPA (200 mg/kg) has been reported to inhibit the spontaneous firing rate of unidentified nerve cells in the cortex (Ostrovskaya and Molodavkin, 1976; Schmutz et al., 1979) and to potentiate intracerebrally applied GABA or muscimol in several regions of the brain (Gent and Phillips, 1980; Kerwin and Olpe, 1980). Only slight effects were found, however, on transynaptic GABA-ergic transmission in the substantia nigra and the nucleus of Deiters (Schmutz et al., 1979; Kerwin et al., 1980).

It has been suggested that DPA exerts its GABA-enhancing effect via inhibition of GABA-T (Godin et al., 1969; Simler et al., 1973). However, it has been reported that DPA inhibits more potently the second enzyme of GABA degradation i.e. SSA-DH (Harvey et al., 1975; Sawaya et al., 1975; Anlezark et al., 1976). Since in the latter reports no explanation was given concerning the mechanism by which GABA concentration was increased, this mechanism has been studied (chapter II).

DPA differs from GABA-T inhibitors with respect to the target system on which they exert their action of enhancing the GABA level. A specific action of DPA in nerve terminals has been demonstrated, whereas GABA-T inhibitors

are active in both nerve terminals and other organelles (Iadarola and Gale, 1979; Sarhan and Seiler, 1979).

In conclusion, DPA exerts its anticonvulsive action probably via inhibition of one of the enzymes involved in GABA degradation leading to an increase in the concentration of GABA in nerve terminals. However, the exact enzymatic mechanism by which the GABA concentration can increase after this inhibition of the degradation, remains to be elucidated.

1.2.7. GABA and behaviour

Systemic injections of GABA, a GABA-agonist or a GABA-T inhibitor into rats have been reported to cause a decrease of spontaneous locomotor activity (Biswas and Carlsson, 1978; Benton and Rich, 1976; Cott et al., 1976; Matsui and Deguchi, 1977; Schechter et al., 1977, 1979; Worms and Lloyd, 1980; Scheel-Krüger et al., 1978 b). In addition, Scotti de Carolis et al. (1978) described a biphasic action after subcutaneous administration of muscimol, viz. a short lasting excitation immediately after injection, followed by muscle relaxation and loss of righting reflex.

Furthermore, intracisternal or intraventricular injection of GABA or the GABA-T inhibitor ethanolamine-O-sulphate (EOS) inhibited spontaneous locomotor activity (Freed and Michaelis, 1976; Czajka, 1978; File, 1977). Thus, either systemic or intracranial injection of GABA-ergic compounds results in sedation of the animals.

However, effects of GABA-ergic compounds on spontaneous behaviour observed after intracerebral injection, depend on the site of injection. Injection of muscimol into the nucleus of the raphé dorsalis, an area containing serotonergic cell bodies, stimulates locomotor activity (Przewlocka et al., 1979). Similarly, injection of GABA agonists into areas containing dopaminergic cell bodies has been found to be excitatory. Tanner (1979) injected GABA into the ventral tegmental area (A 10) -a dopaminergic structure- and found an increase in locomotor activity, which could be antagonized by picrotoxin. However, Mogenson et al. (1979) found an increase in locomotor activity after injection of the GABA antagonist picrotoxin into the ventral tegmental area. This contradiction may be explained by differences in GABA function within the caudal and rostral parts of the ventral tegmental area. Injection of muscimol into the caudal part evokes hypermotility while after such an injection into the rostral part sedation can be observed (Arnt and Scheel-Krüger, 1979).

Injection of muscimol or GABA-T inhibitors into the substantia nigra of rats has been found to evoke stereotype behaviour, including sniffing, biting and gnawing (Scheel-Krüger et al., 1978 a; Matsui and Kamioka, 1978 a, b; Koob et al., 1978). On the other hand, injection of GABA-antagonists into this area elicited catatonia (DiChiara et al., 1978).

Catalepsy and sedation were found after injection of muscimol or the GABA-T inhibitor gabaculine into the nucleus accumbens or into the globus pallidus (Scheel-Krüger et al., 1977, 1978 b; Matsui and Kamioka, 1978, 1979; Wachtel and Andén, 1978; Andén et al., 1978). Catalepsy has also been observed after injection of muscimol into some nuclei of the thalamus (DiChiara et al., 1979).

Thus, in most reports concerning the behavioural effects induced by alteration of central GABA-ergic activity, the relationship with dopaminergic systems has been studied. It may be inferred that injection of GABA-ergic compounds into areas containing dopaminergic cell bodies may cause a stimulation of behavioural activity. Injection of these compounds into areas containing dopaminergic nerve terminals induces sedation and catalepsy.

In conclusion, the enhancement of GABA-ergic transmission *in vivo* has been generally reported to cause sedation. In some reports, excitatory effects were observed. Behavioural effects obtained by modifying GABA-ergic activity in discrete areas of the brain are not fully predictable since both sedative and excitatory effects have been reported.

1.3. MORPHINE ABSTINENCE BEHAVIOUR

1.3.1. Morphine abstinence behaviour, definitions and description

Before describing the symptoms of morphine abstinence behaviour, some terms should be defined. Tolerance is known to develop towards the effects elicited by morphine-like compounds, when these drugs are given repeatedly, while, concomitantly, the animals become physically dependent. Tolerance may be said to have developed when, after repeated administration, a given dose of a drug -in this case morphine- produces a decreased effect. Conversely, tolerance exists when increasingly larger doses must be administered to obtain the effects observed with the original dose. Physical dependence refers to an altered physiological state produced by the repeated administration of a drug, which necessitates the continued administration of the drug to prevent the appearance of a stereotypical

syndrome, the withdrawal or abstinence syndrome characteristic for the particular drug (Jaffe, 1980). The development of tolerance and physical dependence with repeated use is a characteristic feature of all opioid drugs. The degree of tolerance and physical dependence will depend on the particular drug, on the frequency and period of administration and on the quantity administered.

To study morphine abstinence behaviour, morphine is administered chronically to animals, using sustained release preparations, e.g. subcutaneously implanted pellets (Way et al., 1969; Wei et al., 1973 a; Bläsig et al., 1973) or subcutaneously injected emulsions (Collier et al., 1972), multiple injection schedules (Martin et al., 1963) or addition of morphine to the drinking water (Gellert and Holtzmann, 1978). After a sufficiently long period of administration the withdrawal syndrome can be obtained by abrupt cessation of morphine administration or by precipitation,using narcotic antagonists like naloxone, levallorphan or nalorphine.

The symptoms observed in antagonist-precipitated abstinence behaviour in rats have been described by Bläsig et al. (1973). They arranged the symptoms into two classes: counted signs and checked signs. The counted signs were: exploration, jumps, flights, "wet dog shakes" (body shakes), episodes of teeth chattering and writhing. The checked signs were:scream on touch, hostility (irritation) to handling, ptosis, eye twitching, rhinorrhea, lacrimation, diarrhea and penile erection. Other authors have used a slightly modified list, but in principle the abstinence behaviour is described by these symptoms. Wei et al. (1973 a) called jumps and flights "escape attempts" and this notion "escape" can be found also in the "escape digging", observed in sawdust-lined cages used by Frederickson and Smits (1973) and by De Boer et al. (1977) (see also section 1.4.1 and the chapters III, IV and V). The ranking of the relative importance of the symptoms varies from author to author (Wei et al., 1973 a; Collier et al., 1972). However, from the study of Bläsig et al. (1973) it appeared that the relative importance of a symptom depends on the degree of dependency. With a high dependence some signs, e.g. jumps, are dominant, while with a lower degree of dependence more recessive signs, e.g. body shakes, are expressed.

In conclusion, morphine abstinence behaviour consists of various symptoms and the incidence of each particular symptom differs from another and the relative importance of a symptom depends on the degree of dependency.

1.3.2. *Quasi-morphine abstinence behaviour*

In an attempt to unravel the neurochemical and neuropharmacological mechanisms underlying the expression of abstinence symptoms in morphine dependent animals, these animals are often treated with drugs either during the phase of development of dependence or during the phase of withdrawal. The effects of these drugs on the intensity of the withdrawal symptoms is then measured.

However, the mechanism of the induction of morphine abstinence symptoms can also be studied in naive animals which have never received morphine. The advantage of using naive animals is that the experiments can be performed in a short period of time and no interference from a change of morphine level or from the injection of antagonists occurs. Compounds of different categories (Table 1.3.2) can induce in naive animals behavioural symptoms resembling morphine abstinence behaviour. This kind of behaviour has been defined by Collier (1974) as quasi-morphine abstinence behaviour: "An effect resembling one elicited by withdrawal of a drug on which an animal has been made dependent, but produced by another treatment in a naive animal never exposed to drug nor to a like-acting congener that induces such dependence".

For the quasi-abstinence behaviour to be a model for true morphine abstinence behaviour several criteria should be fulfilled. Firstly, the behaviour induced should closely resemble true morphine abstinence behaviour. In several cases,e.g. using phosphodiesterase inhibitors, histamine, DPA, TRH and EGTA, three or more symptoms of the morphine abstinence syndrome have been observed. In other cases, e.g. 5-HTP and kainic acid, also other symptoms occur, i.e. gnawing and biting (5-HTP; Sloviter et al., 1978), and convulsions (kainic acid; Ben-Ari et al., 1979). Therefore, the behaviour induced by the latter two compounds cannot be considered as a specific quasi-morphine abstinence syndrome.

A second criterion is that the effects of morphine on quasi-morphine abstinence behaviour and on true morphine abstinence behaviour should be parallel. One would expect that the quasi-morphine abstinence behaviour can be suppressed by a low dose of morphine. Only the behaviour induced by DPA, by TRH or by the phosphodiesterase inhibitors can be suppressed by morphine, while the effect of morphine has not been studied on responses to EGTA and the opioid compounds. In order to suppress the behaviour induced by 5-HT, ACTH, Ag 3-5 and the Sgd compounds, much higher doses of morphine have been used and in some cases even at these high doses only partial suppression has been found.

The implications of these reports of the effects of phosphodiesterase inhibitors, TRH and EGTA will be discussed briefly. The behaviour induced by DPA is the topic of this thesis and will receive attention in section 1.4.2 and in other chapters.

Phosphodiesterase inhibitors. It has been reported that administration of phosphodiesterase inhibitors or of cyclic AMP during the induction of morphine dependence, accelerates the development of dependence resulting in an intensified abstinence syndrome (Francis et al., 1976; Collier and Francis, 1975; Shahid Salles et al., 1979).These data support the hypothesis by Collier (1978) that cyclic AMP plays a role in morphine abstinence behaviour (Francis et al., 1978 ; for a recent review see Collier, 1980). The implication is that disinhibition of an adenylate cyclase system, which has become tolerant to inhibition by morphine, leads to an overshoot in adenylate cyclase activity during the abstinence phase.

However, the data relating to the role of cyclic AMP in true morphine abstinence behaviour are contradictory. The rise in cyclic AMP in brain, which would be expected to occur according to the afore-mentioned hypothesis, has been found by some authors (e.g. Collier and Francis, 1975), but not by others (VonVoigtlander and Losey, 1977). The increase in cyclic AMP may occur in certain brain areas only, which may explain the conflicting results (Bonnet et al., 1978; for a review see Rosenfeld et al., 1979).

Thyrotropin Releasing Hormone (TRH). The areas of the brain from which body shakes can be elicited by the injection of TRH, namely the medial thalamus, the periaquaductal grey and the locus coeruleus (Wei et al., 1975 a; Kalivas and Horita, 1980), are the same as those from which naloxon injection can precipitate abstinence in morphine pretreated rats (section 1.3.3). Repeated subcutaneous administration of TRH during the induction of morphine dependence in mice has been reported to prevent the development of withdrawal hypothermia, but it failed to modify the jumping response to naloxone (Bhargava, 1980). Administration of TRH prior to precipitation of abstinence by injection of naloxone in morphine dependent rats, exacerbated shaking behaviour while other components of the withdrawal syndrome remain unchanged (Martin et al., 1977). Since TRH is widely distributed in areas of the brain other than the hypothalamus, this endogenous compound may have a direct role in the manifestation of these morphine abstinence symptoms which are related to the thermoregulatory system (Jackson and Reichlin, 1974; Winoku and Utiger, 1974).

EGTA. Since EGTA is pre-eminently a chelator of calcium, the reports cited indicate an important role for calcium in morphine abstinence behaviour. This role of calcium in morphine abstinence behaviour appears to be intimately related to neurotransmitter release since the concentration of calcium in synaptic vesicles has been found to be particularly affected by morphine (for reviews see Way, 1978; Ross and Cardenas, 1979). Administration of morphine acutely lowers vesicular calcium, while, during the development of tolerance and dependence, an accumulation of vesicular calcium occurs. The sudden removal of morphine leads to increased release of neurotransmitters resulting in the hyperactivity of several neurotransmitter systems, which characterizes morphine withdrawal behaviour (Way, 1978).

Although the syndromes induced by 5-HTP or by amphetamine and L-DOPA do not seem to be as closely related as the afore-mentioned syndromes to quasi-morphine abstinence behaviour, the data do suggest that an overshoot of serotonergic and catecholaminergic activity may form a part of the underlying mechanism in the morphine abstinence behaviour. Such an enhanced neuronal activity may become apparent in various ways. From enhancement of the release of the transmitter, as has been suggested in relation to the response to EGTA or from an enhanced sensitivity of the receptors or from a decrease in reuptake or extra neuronal degradation of the transmitters. An altered receptor sensitivity has already been suggested as the mechanism underlying abstinence behaviour (Collier, 1965; Jaffe and Sharpless, 1968). In recent years, receptor binding studies have confirmed this theory, e.g. for β-adrenergic receptors (Llorens et al., 1978). No data exist to support the involvement of decreased uptake or degradation. In contrast, an enhanced turnover of noradrenaline appears to be present during morphine abstinence (Laverty and Roth, 1980; Crawley et al., 1979).

It is not within the scope of this chapter to discuss all the implications of the data summarized in Table 1.3.2. However, it is clear that when studying quasi-morphine abstinence behaviour, data are yielded which support the research on true morphine abstinence behaviour.

In conclusion, it has been shown that quasi-morphine abstinence behaviour can be induced by interference at several levels of brain function, i.e. at the level of transmitter release or receptor stimulation, or at the level of the second messenger cyclic AMP.

Table 1.3.2. Compounds inducing quasi-morphine abstinence behaviour

Category	Individual compounds	R.o.a.	Signs induced	Morphine Dose	Morphine Effect	Reference
Phosphodiesterase inhibitors						
	IBMX	s.c.	Jumping, body shakes, diarrhea, irritability	(1.0)[5]	↓	1,2
	Theophylline	s.c.				3,4
	Caffeine	s.c.				5
Precursors or transmitter agonists						
	5-HTP	i.p.	Body shakes, irritability	n.t.		6
	5-HTP + carbidopa	s.c./i.p.	Body shakes	n.t.		7
	5-HTP + benserazide	i.p./i.p.	Body shakes	n.t.		8
	5-HTP + carbidopa	i.p./i.p.	Body shakes	n.t.		9
	5-HT	i.c.v.	Body shakes	10	↘	10
	Amphetamine + L-DOPA	i.p./i.p.	Jumping	n.t.		11
	Histamine	i.c.[1]	Body shakes, teeth chattering, irritability	n.t.		12
	DPA	i.p.	Body shakes, escape digging, piloerection, ptosis	1.0	↓	13
	Kainic acid	i.c.v.	Body shakes, teeth chattering, salivation, chewing	4.0	-	14
	Kainic acid	i.c.[2]	Body shakes, chewing, paw tremor	n.t.		15
Peptides						
	TRH	i.v.	Body shakes, tremor	n.t.		16
	TRH	i.c.v.	Body shakes, paw tremor, lacrimation	n.t.		17
	TRH	i.p.	Body shakes	2.0	↓	18
	TRH	i.c.[3]	Body shakes, tremor, lacrimation	n.t.		19,20
	Met-enkephalin	i.c.v.	Body shakes	n.t.		21
	β-endorphin	i.c.v.	Jumping, body shakes, diarrhea	n.t.		22,23
	ACTH	i.c.[4]	Jumping, teeth chattering, body shakes, irritability	30	↓	24

Category	Individual compounds	R.o.a.	Signs induced	Morphine Dose	Morphine Effect	Reference
Opioid compounds						
	RX-336-M	i.p.	Body shakes, ptosis, tremor	1.0	↓	25,34
	Pentazocine	i.p.	Body shakes	n.t.		26
	Ketocyclazocine	i.c.v.	Body shakes	n.t.		27
	Morphine/Morphine-glucuronide	i.c.v.	Body shakes	n.t.		28
Miscellaneous agents						
	Sgd 8473/15073	i.p.	Body shakes, tremor, escape behaviour, ptosis	50	↘	29,34
	Ag 3-5	i.p.	Body shakes, tremor, escape behaviour, ptosis	10	↓	30,34
	Steroid-O-sulphate	i.c.v.	Body shakes, salivation, irritability	n.t.		31
	Androsteronsulphate	i.c.v.	Body shakes, escape behaviour	n.t.		32
	EGTA	i.c.v.	Body shakes, salivation, irritability	n.t.		31
	EGTA	i.c.v.	Body shakes, escape behaviour, weight loss	n.t.		33

Abbreviations: R.o.a. - route of administration; s.c. - subcutaneously; i.p. - intraperitoneally; i.c.v. - intracerebroventricularly; i.v. - intravenously; i.c. - intracerebrally; 1) dorsal hippocampus; 2) amygdala; 3) hypothalamus; thalamus, preoptic area, locus coeruleus; 4) periaquaductal grey; 5) heroine. ↓ = nearly total suppression; ↘ = only partial suppression; - = no effect; n.t. = not tested. References: 1) Francis et al., 1975; 2) Francis et al., 1976; 3) Collier et al., 1974; 4) Francis et al., 1978; 5) Butt et al., 1979; 6) Collier, 1974; 7) Bedard and Pycock, 1977; 8) Sloviter et al., 1978; 9) Fozard and Palfreyman, 1979; 10) Drust et al., 1979; 11) Lal et al., 1975; 12) Glick and Crane, 1978; 13) De Boer et al., 1977; 14) Lanthorn and Isaacson, 1978; 15) Ben-Ari et al., 1979; 16) Schenkel-Hülliger et al., 1974; 17) Prange et al., 1974; 18) Martin et al., 1977; 19) Wei et al., 1975; 20) Kalivas and Horita, 1980; 21) Leybin et al., 1976; 22) Gispen et al., 1976; 23) Bloom et al., 1976; 24) Jaquet et al., 1978; 25) Cowan and Watson, 1978; 26) Schneider, 1968; 27) Lanthorn et al., 1979; 28) LaBella et al., 1979 b; 29) Jahn and Mixich, 1976; 30) Wei, 1976; 31) LaBella et al., 1979 a; 32) LaBella et al., 1978; 33) Schmidt and Way, 1980; 34) Cowan, 1981.

1.3.3. *Localization of sites involved in abstinence behaviour*

Localization of the areas involved in morphine abstinence behaviour is performed by many authors by lesioning the brain or by injection of opiate antagonists into particular sites of the brain in morphine dependent animals. The lesion technique, however, has several drawbacks since it is not possible to decide whether a lesion was truly made at the primary site responsible for withdrawal signs. The lesioned brain structure may be merely interconnected with the primary site. Therefore, lesion studies should always be accompanied by injection studies using injections of different substances into the same structure. Taking these precautions into consideration, no clear conclusions have been drawn from lesion studies in morphine withdrawal (see Pert, 1978).

Results of the studies using the antagonist microinjection technique in rats are summarized in Table 1.3.3. The studies of Laschka et al. (1976) and Laschka and Herz (1977) were carried out using labelled levallorphan or naloxone and the distribution of the label was studied autoradiographically. Aghajanian (1978) reported that the locus coeruleus, probably the most important structure in the anterior part of the floor of the 4th ventricle with respect to the induction of abstinence signs (Laschka et al., 1976), exhibits a hyperactive firing rate after precipitation of abstinence, a finding which is in agreement with the enhanced noradrenaline turnover mentioned in the preceding section. The involvement of the amygdala, as suggested by Lagowska et al. (1978) and Calvino et al. (1979), is supported by the finding that β-endorphin-induced body shakes occurred immediately after high frequency bursts recorded from the amygdala, bursts which were not observed in the hippocampus or the cortex (Henriksen et al., 1978).

The approach opposite to that of the antagonist microinjection method has been used by Wei et al. (1975c), who studied the sites at which morphine suppresses body shakes, evoked by immersion of anaesthetized rats into cold water. Three sites, i.e. the locus coeruleus, the medial preoptic area and the periaquaductal grey, appeared to be highly sensitive to morphine, whereas no effect could be found on injecting morphine into the amygdala. These areas, apart from the amygdala, are similar to those which were found to be sensitive to naloxone in morphine-dependent rats (see Table 1.3.3).

Thus, the occurrence of morphine abstinence symptoms is related to several brain areas, i.e. locus coeruleus, periaquaductal grey, medial preoptic area, medial thalamus and amygdala. However, apart from the locus

coeruleus no specific transmitter system has yet been implicated in any of these brain structures.

Table 1.3.3. Precipitated abstinence by intracerebral injection of morphine antagonists in the rat.

Antagonist	Dose (µg)	Site of action	Ref.
Naloxone	40-200	medial thalamus, diencephalic-mesencephalic junctures	1,2
Naloxone	1.5	medial hypothalamus, medial preoptic area, periaquaductal grey, 4th ventricular spaces	3
Levallorphan	1.5-25	anterior part of the floor of the 4th ventricle	4
Naloxone	0.4- 6.0	locus coeruleus, medial thalamus, periaquaductal grey	
Naloxone	1.5	floor of the 4th ventricle, periaquaductal grey	5
Naloxone	40	amygdala, striatum	6
Naloxone	10	several nuclei of the amygdala, striatum	7

References: 1) Wei et al., 1972; 2) Wei et al., 1973 b; 3) Wei et al., 1975 b; 4) Laschka et al., 1976; 5) Laschka and Herz, 1977; 6) Lagowska et al., 1978; 7) Calvino et al., 1979.

In conclusion, antagonist microinjection studies in morphine dependent animals indicate that abstinence symptoms are mediated by more than one area of the brain.

1.3.4. Morphine actions on the GABA-system

Reports on the acute effects of morphine on GABA levels in the brain are scarce and the results are not clear either. Decreases and increases in GABA concentration in the brain have both been found (Lin et al., 1973; Takanaka et al., 1976), although other authors could not find any change in GABA after administration of morphine (Maynert et al., 1962; Seethy and Bombardt, 1978). Regional differences in the effect of morphine on the GABA level, however, have been described and may explain these different results (Kuschinsky et al., 1976; Moroni et al., 1978, 1979; Costa et al., 1978).

Chronic treatment of mice with morphine has been reported to decrease whole brain GABA level as well as GAD activity (Takanaka et al., 1976). In addition, after morphine withdrawal in rats, a decreased level of GABA in the cerebellum and an increased GABA-concentration in the hypothalamus

has been observed (Lin et al., 1973; Tzeng and Ho, 1978; Volicer et al., 1977).

With respect to the GABA shunt enzymes, only minor effects of morphine on GABA-T have been reported, whereas a decrease in the activity of GAD is found after chronic morphine administration (Tzeng and Ho, 1978), possibly due to a decreased affinity of this enzyme for PLP. This decrease can be reversed by withdrawal of morphine or by precipitating abstinence with naloxone (Ho and Gilliland, 1979).

The interaction between morphine and GABA has also been studied at the cellular level. Morphine has no influence on the inhibitory effect of exogenously applied GABA in the hippocampus (Segal, 1977). However, morphine was reported to decrease the inhibitory effects of putative GABA-ergic pathways in this area, presumably via a presynaptic mechanism (Corrigall and Linseman, 1980). The presence of a presynaptic effect of morphine on GABA-neurons is supported by the fact that morphine inhibits the potassium-evoked release of GABA *in vivo* and *in vitro* (Iwatsubo and Kondo, 1978; Van der Heyden et al., 1980; Coutinho-Netto et al., 1980).

At the receptor level there also seems to be a relationship between GABA and opiates, since GABA—antagonistic features have been ascribed to naloxone, morphine, levorphanol and dextrorphan (Breuker et al., 1976; Dingledine et al., 1977). These data are relevant only when high doses of these compounds will be used (see Gruol et al., 1980).

Thus chronic administration of morphine appears to result in a decreased GABA-ergic activity either by a decrease of the activity of GAD or by inhibition of the release. Administration of naloxone or withdrawal of morphine will then result in a relative increase in GABA-ergic activity.

In conclusion, the interaction of morphine with the GABA-ergic system *in vivo* is complex, possibly due to regional differences. In most cases morphine decreases GABA-ergic activity.

1.4. DPA-INDUCED QUASI-MORPHINE ABSTINENCE BEHAVIOUR

1.4.1. Methods

All experiments were performed on male albino Wistar rats (TNO, Zeist, The Netherlands). At the start of the experiment rats were allowed to habituate for at least 30 min in a transparant Macrolon cage (47x27x15 cm) with sawdust bedding. Thereafter an intraperitoneal injection of DPA was

given (in most cases 300 mg/kg at a concentration of 100 mg/ml deionized water). Each rat was continuously observed for the following 15 min, subdivided into three periods of 5 min, and its locomotor activity monitored using a Varimex activity meter, equipped for alternating measurement of horizontal and vertical activity every 10 sec. The following signs were checked as absent, mild or marked: salivation, rhinorrhea, lacrimation, urination, diarrhea, penile erection and ejaculation, ptosis, teeth chattering, swallowing, tremor, hunchback posture, piloerection, irritability to handling and reaction on poking. Other signs were counted as quantitative events: escape digging, body shakes, head shakes, foreleg shakes and yawning.

SCORING ABSTINENCE USING THE SYSTEM OF FREDERICKSON AND SMITH.

ITEM	0 - 5 min	5 - 10 min	10 - 15 min	0 - 15 min
SALIVATION				
RHINORRHEA				
LACRIMATION				
URINATION				
DIARRHEA				
ERECTION				
EJACULATION				
PTOSIS				
TEETH CHATTER				
SWALLOWING				
TREMOR				
HUNCHBACK POSTURE				
PILOERECTION				
IRRITATION HANDL.				
REACTION POKING				

ESCAPE DIGGING				
ESCAPE JUMPING				
WET DOG SHAKES				
HEAD SHAKES				
FORELEG SHAKES				
YAWNING				
TOTAL SCORE				

MOTOR ACTIVITY	0 - 5 min	5 - 10 min	10 - 15 min	0 - 15 min
HORIZONTAL ACTIVITY				
VERTICAL ACTIVITY				
TOTAL ACTIVITY				
HOR./ VERT. RATIO				

ANIMAL	
TREATMENT	
WEIGHT	
DATE	
GENERAL REMARKS	

FIG. 1.4.1. Scoring-sheet used for measuring DPA-induced abstinence behaviour. From De Boer (1977).

From this checklist (Fig. 1.4.1) of behavioural symptoms described by Frederickson and Smits (1973), an abstinence score was calculated, according to these authors, in which the checked signs received 0, 2 or 4 when they were absent, mild or marked, respectively, and the counted signs received 0, 2, 4, 6, 8, or 10 when they occurred 2-5, 6-10, 11-20, 21-40 or more than 40 times per 15 min period, respectively. Table 1.4.1 gives a characterization of the DPA-induced behaviour. Not all signs of morphine abstinence behaviour occur after injection of DPA, but the signs present after this treatment can be summarized as "central signs" (Frederickson,1975).

Table 1.4.1. Characterization of DPA-induced behaviour

Item	Treatment		Significance
	DPA	Saline	p
A. Incidence of:			
Erection	21	5	< 0.1
Teeth chatter	16	0	< 0.1
Swallowing	32	0	< 0.01
Tremor	5	0	-
Hunchback posture	95	0	< 0.001
Piloerection	89	11	< 0.001
Irritation	5	0	-
Digging	68	21	< 0.001
Body shaking	95	5	< 0.001
Head shaking	21	5	-
Foreleg shaking	21	0	0.053
Catalepsy	42	0	< 0.005
B. Frequency of:			
Body shaking	19.7 ± 2.1	0.2 ± 0.1	< 0.001
Digging	10.6 ± 2.9	1.1 ± 0.5	< 0.001
C. Total abstinence score	17.3 ± 1.0	1.2 ± 0.4	< 0.001
D. Activity:			
Horizontal activity	252 ± 16	52 ± 10	< 0.001
Vertical activity	347 ± 24	195 ± 30	< 0.001

Summary of the DPA-induced abstinence behaviour for 19 rats treated with 300 mg/kg DPA intraperitoneally and being observed for 15 min. A symptom was considered to be present when it contributed to the total abstinence score. The incidence of a particular behavioural item refers to the percentage of the treated rats exhibiting that abstinence symptom, while the frequency of shaking or digging represents the number of events per 15 min observation period. Also, activity parameters are presented as counts per 15 min period. Statistical significance was calculated using Fisher's exact probability test (A) or the Mann-Whitney U-test (B, C and D).
From De Boer et al. (1980)

1.4.2. *The role of GABA*

In this section the behavioural syndrome induced by DPA will be described with respect to the pharmacological aspects, summarizing the results of De Boer (1977).

A role for GABA in this behaviour is suggested by the action of DPA to increase the concentration of GABA in the brain (see section 1.2.6) and by the suppression of the behaviour by treatment with subconvulsive doses of the GABA antagonists bicuculline (De Boer et al., 1977) and picrotoxin (De Boer et al., 1980). The GABA synthesis inhibitor, 3-mercaptopropionic acid, has also been found to antagonize DPA-induced behaviour, while strychnine, a convulsant glycine antagonist, has no effect. These data indicate that the DPA-induced behaviour may be a correlate of increased GABA-ergic activity (De Boer et al., 1980).

This behavioural syndrome has also been compared with behavioural effects obtained after injection of the GABA-T inhibitor aminooxyacetic acid (AOAA). Administration of AOAA does not induce abstinence behaviour, although this treatment is known to enhance the GABA concentration in the brain (Wallach, 1961). Moreover, DPA-induced abstinence behaviour is suppressed in AOAA-pretreated rats, indicating that GABA-T inhibition counteracts rather than potentiates DPA-induced abstinence behaviour.

It has, therefore, been postulated that DPA and GABA-T inhibitors may act in two different GABA compartments with opposite roles, in relation to the behaviour studied (De Boer, 1977; Fig. 1.4.2).

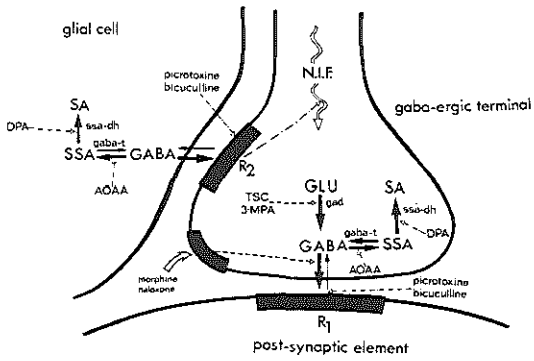

FIG. 1.4.2. A model of the actions of morphine and GABA-ergic drugs on a GABA neuron. Two types of GABA receptors are proposed: Type R_1, mediating abstinence behaviour and located on a postsynaptic element, and type R_2, GABA-sensitive pre-synaptic inhibitory auto-receptors affected by release of GABA from glial cells due to inhibition of GABA degradation by by AOAA. The effect of morphine, preventing the release of GABA, might be mediated via opiate receptors, located on the pre-synaptic element. From De Boer et al. (1980).

Abbreviations used: GLU - glutamate; SA - succinate; GAD - glutamate decarboxylase; GABA-T - GABA transaminase; SSA-DH - SSA dehydrogenase; N.I.F. - nerve impulse flow; ----→ - inhibition.

Accordingly, DPA increased GABA concentration only in nerve endings, initiating quasi-abstinence behaviour, while GABA-T inhibitors will mainly prevent GABA degradation in glial cells (see section 1.2.6). Consequently, GABA, leaking or released from glial cells, may act on presynaptic GABA receptors. This effect on presynaptic GABA receptors will cause a decreased GABA-ergic activity impairing quasi-abstinence behaviour. The specificity of DPA-induced quasi-morphine abstinence behaviour as a model for true morphine abstinence behaviour has been discussed in section 1.3.2.

In conclusion, DPA-induced behaviour can be considered as a possible correlate of GABA-ergic activity and it is a specific quasi-morphine abstinence syndrome.

1.4.3. Aim of this thesis

In the previous section it was suggested that DPA induces quasi-morphine abstinence behaviour via an increased stimulation of GABA receptors. It is probable that this increased GABA-ergic stimulation occurs via inhibition of the degradation of GABA (section 1.2.6). Since the mechanism by which DPA increases the GABA concentration had not been clearly characterized, it was our aim to study this mechanism and to examine its possible contribution to explain the induction of quasi-morphine abstinence behaviour.

The behaviour observed after administration of DPA may throw some light on the relation between GABA and morphine-abstinence behaviour. It enabled us to study the involvement of particular GABA-ergic systems in the brain in the induction of abstinence behaviour.

In brief, the aim of our research was to characterize the mechanism responsible for the behaviour evoked by DPA, to locate its site of action, and to obtain, in this way, a deeper understanding of possible mechanisms involved in morphine abstinence behaviour.

In this thesis experiments are described to specify the role of the GABA-ergic system in the mechanism of action of DPA (chapter II). Using analogues of DPA we then studied the involvement of this mechanism of action in the quasi-abstinence behaviour (chapter III). The discrepancy between the time course of the behavioural effects of DPA and of its effect on the level of GABA in brain, known from the literature, was studied (chapter IV). Finally, the involvement of several GABA-containing brain structures in the DPA-induced behaviour was studied in order to characterize the site of action of DPA in evoking this behaviour (chapters V and VI).

REFERENCES

Abe, M., Matsuda, M. (1976) The role of γ-aminobutyrate metabolism in the mechanism of convulsions. Jikeikai Med. J. 23, 245-253

Abe, M., Matsuda, M. (1977) γ-Aminobutyric acid metabolism in subcellular particles of mouse brain and its relationship to convulsions. J. Biochem. Tokyo, 82, 195-200

Aghajanian, G.K. (1978) Tolerance of locus coeruleus neurons to morphine and suppression of withdrawal response by clonidine. Nature 276, 186-188

Albers, R.W., Koval, G.J. (1961) Succinic semialdehyde dehydrogenase: purification and properties of the enzyme from monkey brain. Biochim. Biophys. Acta 52, 29-35

Andén, N.-E., Grabowska-Andén, M., Wachtel, H. (1978) Effects of GABA and GABA-like drugs on the brain dopamine and on the motor activity of rats. In: GABA-Neurotransmitters. Pharmacochemical, Biochemical and Pharmacological aspects (Krogsgaard-Larsen, P., Scheel-Krüger, J., Kofod, H., eds) Munksgaard, Copenhagen, pp. 135-146

Anlezark, G., Collins, J., Meldrum, B. (1978) GABA-agonists and audiogenic seizures. Neurosci. Lett. 7, 337-340

Anlezark, G.M., Horton, R.W., Meldrum, B.S., Sawaya, M.C.B. (1976) Anticonvulsant action of ethanolamine-O-sulphate and di-n-propylacetate and the metabolism of γ-aminobutyric acid (GABA) in mice with audiogenic seizures. Biochem. Pharmacol. 25, 413-417

Arnt, J., Scheel-Krüger, J. (1979) GABA in the ventral tegmental area: differential regional effects on locomotion, aggression and food intake after microinjection of GABA agonists and antagonists. Life Sciences 25, 1351-1360

Awapara, J., Landua, A.J., Fuerst, R., Seale, B. (1950) Free γ-aminobutyric acid in brain. J. Biol. Chem. 187, 35-39

Balázs, R., Cremer, J.E. (eds) (1972) Metabolic compartmentation in the brain. MacMillan, London 383 pp.

Balázs, R., Dahl, D., Harwood, J.R. (1966) Subcellular distribution of enzymes of glutamate metabolism in rat brain. J. Neurochem. 13, 897-895

Balázs, R., Machiyama, Y., Patel, A.J. (1972 a) Compartmentation and the metabolism of γ-aminobutyrate. In: Metabolic compartmentation in the brain (Balázs, R., Cremer, J.E., eds) MacMillan, London, pp. 57-70

Balázs, R., Patel, A.J., Richter, D. (1972 b) Metabolic compartments in the brain: their properties and relation to morphological structures. In: Metabolic compartmentation in the brain (Balázs, R., Cremer, J.E., eds) MacMillan, London, pp. 167-184

Balcom, G.J., Lenox, R.H., Meyerhoff, J.L. (1975) Regional γ-aminobutyric acid levels in rat brain determined after microwave fixation. J. Neurochem. 24, 609-613

Barber, R., Saito, K. (1976) Light microscopic visualization of GAD and GABA-T in immunocytochemical preparations of rodent CNS. In: GABA in nervous system function (Roberts, E., Chase, T.N., Tower, D.B., eds) Raven, New York, pp. 113-132

Baxter, C.F. (1976) Some recent advances in studies of GABA metabolism and compartmentation. In: GABA in nervous system function (Roberts, E., Chase, T.N., Tower, D.B., eds) Raven, New York, pp. 61-87

Bayón, A., Possani, L.D., Rode, G., Tapia, R. (1978) Kinetics of brain glutamate decarboxylase. Dead-end and product inhibition studies. J. Neurochem. 30, 1629-1631

Bedard, P., Pycock, C. (1977) 'Wet dog' shake behaviour in the rat: a possible

quantitative model of central 5-hydroxytryptamine activity. Neuropharmacology 16, 663-670
Ben-Ari, Y., Lagowska, J., Tremblay, E., LeGal La Salle, G. (1979) A new model of focal status epilepticus: intra-amygdaloid application of kainic acid elicits repetitive secondarily generalized convulsive seizures. Brain Research 163, 176-179
Benton, D., Rick, J.T. (1976) The effect of increased brain GABA produced by amino-oxyacetic acid on arousal in rats. Psychopharmacology 49, 85-89
Berl, S., Clarke, D.D., Schneider, D. (eds) (1975) Metabolic compartmentation and Neurotransmission: Relation to structure and function in brain. Plenum, New York, 721 pp.
Bernheimer, H., Horniekiewicz, O. (1962) Das Verhalten einiger Enzyme im Gehirn normaler und Parkinson-kranker Menschen. Naunyn-Schmied. Arch. Exp. Path. Pharmakol. 243, 295-296
Bhargava, H.N. (1980) The effects of thyrotropin-releasing hormone on the central nervous system responses to chronic morphine administration. Psychopharmacology 68, 185-189
Bird, E.D., Mackay, A.V.P., Rayner, C.N., Iversen, L.L. (1973) Reduced glutamic-acid-decarboxylase activity of *post-mortem* brain in Huntington's chorea. Lancet 1, 1090-1092
Biswas, B., Carlsson, A. (1978) Effect of intraperitoneally administered GABA on the locomotor activity in mice. Psychopharmacology 59, 91-94
Bläsig, J., Herz, A., Reinhold, K., Zieglgänsberger, S. (1973) Development of physical dependence on morphine in respect to time and dosage and quantification of the precipitated withdrawal syndrome in rats. Psychopharmacologia 33, 19-38
Blindermann, J.M., Maitre, M., Ossola, L., Mandel, P. (1978) Purification and some properties of L-glutamate decarboxylase from human brain. Europ. J. Biochem. 86, 143-152
Bloch-Tardy, M., Rolland, M., Gonnard, P. (1974) Pig brain 4-aminobutyrate 2-ketoglutarate transaminase. Purification, kinetics and physical properties. Biochimie 56, 823-832
Bloom, F.E., Segal, D., Ling, N., Guillemin, R. (1976) Endorphins: profound behavioral effects in rats suggest new etiological factors in mental illness. Science 194, 630-632
Blume, H.W., Lamour, Y., Arnauld, E., Layton, B.S., Renaud, L.P. (1979) Sodium di-n-propylacetate (valproate) action on single neurons in rat cerebral cortex and hippocampus. Brain Research 171, 182-185
Bonnet, K.A., Gusik, S.A., Sunshine, A.G. (1978) Multiple opiate receptors reflected in region specific alterations in brain cyclic nucleotides. In: Characteristics and function of opioids (Van Ree, J.M., Terenius, L., eds) Elsevier/North Holland, Amsterdam, pp.453-464
Braganca, B.M., Faulkner, P., Quastel, J.H. (1953) Effects of inhibitors of glutamine synthesis on inhibition of acetylcholine synthesis in brain slices by ammonium ions. Biochim. Biophys. Acta 10, 83-88
Brennan, M.J.W., Cantrill, R.C., Oldfield, M., Krogsgaard-Larsen, P. (1981) Inhibition of γ-aminobutyric acid release by γ-aminobutyric acid agonist drugs. Molec. Pharmacol. 19, 27-30
Breuker, E., Dingledine, R., Iversen, L.L. (1976) Evidence for naloxone and opiates as GABA antagonists. Br. J. Pharmacol. 58, 458P
Butt, N.M., Collier, H.O.J., Cuthbert, N.J., Francis, D.L., Saeed, S.A. (1979) Mechanism of quasi-morphine withdrawal behaviour induced by methylxanthines. Europ. J. Pharmacol. 53, 375-378

Calvino, B., Lagowska, J., Ben-Ari, Y. (1979) Morphine withdrawal syndrome: differential participation of structures located within the amygdaloid complex and striatum of the rat. Brain Research 177, 19-34
Cash, C., Cieselski, L., Maitre, M., Mandel, P. (1975) Purification de la semi-aldéhyde succinique déshydrogenase de cerveau de rat et étude de son inhibition par des acides gras ramifiés. C.R. Soc. Biol. (Paris) 169, 884-887
Cash, C., Cieselski, L., Maitre, M., Mandel, P. (1978) Purification and properties of rat brain succinic semialdehyde dehydrogenase. Biochimie 59, 257-268
Chan-Palay, V., Wu, J.-Y., Palay, S.L. (1979) Immunocytochemical localization of γ-aminobutyric acid transaminase at cellular and ultrastructural levels. Proc. Nat. Acad. Sci. U.S.A. 76, 2067-2071
Cieselski, L., Maitre, M., Cash, C., Mandel, P. (1975) Regional distribution in brain and effect on cerebral mitochondral respiration of the anti-convulsive drug n-dipropylacetate. Biochem. Pharmacol. 24, 1055-1058
Collier, H.O.J. (1965) A general theory of the genesis of drug dependence by induction of receptors. Nature 205, 181-182
Collier, H.O.J. (1974) The concept of the quasi-abstinence effect and its use in the investigation of dependence mechanisms. Pharmacology 11, 58-61
Collier, H.O.J. (1978) Biochemical theories of opioid dependence: An analysis. Proc. Eur. Soc. Neurochem. 1, 374-385
Collier, H.O.J. (1980) Cellular site of opiate dependence. Nature 283, 625-629
Collier, H.O.J., Francis, D.L. (1975) Morphine abstinence is associated with increased brain cyclic AMP. Nature 255, 159-162
Collier, H.O.J., Francis, D.L., Henderson, G., Schneider, C. (1974) Quasi-morphine abstinence syndrome. Nature 249, 471-473
Collier, H.O.J., Francis, D.L., Schneider, C. (1972) Modification of morphine withdrawal by drugs interacting with humoral mechanisms: some contradictions and their interpretation. Nature 237, 220-223
Corrigall, W.A., Linseman, M.A. (1980) A specific effect of morphine on evoked activity in the rat hippocampal slice. Brain Research 192, 227-238
Costa, E., Fratta, W., Hong, J.S., Moroni, F., Yang, H.-Y.T. (1978) Interactions between enkephalinergic and other neuronal systems. In: The endorphins. Adv. Biochem. Psychopharmacol. Vol. 18 (Costa, E., Trabucchi, M., eds) Raven, New York, pp. 111-123
Cott, J.M., Carlsson, A., Engel, J., Lindquist, M. (1976) Suppression of ethanol-induced locomotor stimulation by GABA-like drugs. Naunyn-Schmied. Arch. Pharmacol. 295, 203-209
Coutinho-Netto, J., Abdul-Ghani, A.S., Bradford, H.F. (1980) Suppression of evoked and spontaneous release of neurotransmitters *in vivo* by morphine. Biochem. Pharmacol. 29, 2777-2780
Cowan, A. (1981) RX 336-M, a new chemical tool in the analysis of the quasi-morphine withdrawal syndrome. Fed. Proc. 40, 1497-1501
Cowan, A., Watson, T. (1978) Lysergic acid diethylamide antagonizes shaking induced in rats by five chemically different compounds. Psychopharmacology 57, 43-46
Crawley, J.N., Laverty, R., Roth, R.H. (1979) Clonidine reversal of increased norepinephrine metabolite levels during morphine withdrawal. Europ. J. Pharmacol. 57, 247-250
Czajka, R. (1978) Central effects of endogenous and exogenous GABA. Acta Physiol. Pol. 29, 193-205

De Boer, T. (1977) GABA shunt enzymes and the relationship with morphine abstinence. Ph.D. Thesis, Erasmus University, Rotterdam
De Boer, T., Bruinvels, J. (1977 a) Assay and properties of 4-aminobutyric-2-oxoglutaric acid transaminase and succinic semialdehyde dehydrogenase in rat brain tissue. J. Neurochem. 28, 471-478
De Boer, T., Bruinvels, J. (1977 b) Succinic semialdehyde dehydrogenase: activation by monovalent and divalent cations. Proc. Int. Soc. Neurochem. 6, 524
De Boer, T., Bartels, K., Metselaar, H.J., Bruinvels, J. (1980) Di-n-propylacetate induced abstinence behaviour as a possible correlate of increased GABA-ergic activity in the rat. Psychopharmacology 71 (1980) 257-267
De Boer, T., Bruinvels, J., Bonta, I.L. (1979) Differential effects of GABA analogues and zinc on glutamate decarboxylase, 4-aminobutyric-2-oxoglutaric acid transaminase and succinate semialdehyde dehydrogenase in rat brain tissue. J. Neurochem. 33, 597-601
De Boer, T., Metselaar, H.J., Bruinvels, J. (1977) Suppression of GABA-induced abstinence behaviour in naive rats by morphine and bicuculline. Life Sciences 20, 933-942
De Feudis, F.V. (1978) Vertebrate GABA receptors. Neurochem. Res. 3, 263-280
DiChiara, G., Morelli, M., Porceddu, M.L., Gessa, G.L. (1978) Evidence that nigral GABA mediates behavioural responses elicited by striatal dopamine receptor stimulation. Life Sciences 23, 2045-2052
DiChiara, G., Morelli, M., Porceddu, M.L., Gessa, G.L. (1979) Role of thalamic γ-aminobutyrate in motor functions: catalepsy and ipsiversive turning after intra-thalamic muscimol. Neuroscience 4, 1453-1465
Dingledine, R., Iversen, L.L., Breuker, E. (1978) Naloxone as a GABA antagonist: evidence from iontophoretic, receptorbinding and convulsant studies. Europ. J. Pharmacol. 47, 19-27
Douglas, W.W. (1968) Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. Br. J. Pharmacol. 34, 451-474
Dravid, A.R., Jilek, J. (1965) Influence of stagnant hypoxia (oligaemia) on some free amino acids in rat brain during ontogeny. J. Neurochem. 12, 837-843
Drust, E.G., Sloviter, R.S., Connor, J.D. (1979) Effect of morphine on 'wet dog' shakes caused by cerebroventricular injection of serotonin. Pharmacology 18, 299-305

Embree, L.J., Albers, R.W. (1964) Succinic semialdehyde dehydrogenase from human brain. Biochem. Pharmacol. 13, 1209-1217
Enna, S.J., Ferkany, J.W., Strong, R. (1980) Drug-induced alterations in neurotransmitter receptorbinding and function. In: Receptors for neurotransmitters and peptide hormones (Pepeu, G., Kuhar, M.J., Enna, S.J., eds) Adv. Biochem. Psychopharmacol. Vol. 21, Raven, New York pp. 253-263
Enna, S.J., Snyder, S.H. (1975) Properties of γ-aminobutyric acid (GABA) receptor-binding in rat brain synaptic membrane fractions. Brain Research 100, 81-97

Fagg, G.E., Lane, J.D. (1979) The uptake and release of putative amino acid neurotransmitters. Neuroscience 4, 1015-1036
File, S.E. (1977) Raised brain GABA levels, motor activity and exploration in the rat. Brain Research 131, 180-183
Fonnum, F., Grofova, I., Rinvik, E., Storm-Mathisen, J., Walberg, F. (1974) Origin and distribution of glutamate decarboxylase in the substantia nigra of the cat. Brain Research 171, 77-92

Fonnum, F., Walberg, F. (1973 a) An estimation of the concentration of γ-aminobutyric acid and glutamate decarboxylase in the inhibitory Purkinje axon terminals in the cat. Brain Research 54, 115-127
Fonnum, F., Walberg, F. (1973 b) The concentration of GABA within inhibitory nerve terminals. Brain Research 62, 577-579
Fozard, J.R., Palfreyman, M.G. (1979) Metoclopramide antagonism of 5-hydroxytryptophan-induced "wet dog" shake behaviour in the rat. Naunyn-Schmied. Arch. Pharmacol. 307, 135-142
Francis, D.L., Cuthbert, N.J., Dinneen, L.C., Schneider, C., Collier, H.O.J. (1976) Methylxanthine-accelerated opiate dependence in the rat. In: Opiates and endogenous opioid peptides. (Kosterlitz, H.W. ed) Elsevier/North Holland, Amsterdam pp.177-184
Francis, D.L., Cuthbert, N.J., Saeed, S.A., Butt, N.M., Collier, H.O.J. (1978) Meaning of quasi-withdrawal phenomena for the cellular mechanism of abstinence. In: Characteristics and function of opioids. (Van Ree, J.M., Terenius, L., eds) Elsevier/North Holland, Amsterdam pp. 37-49
Francis, D.L., Roy, A.C., Collier, H.O.J. (1975) Morphine abstinence and quasi-abstinence effects after phosphodiesterase inhibitors and naloxone. Life Sciences 16, 1901-1906
Frederickson, R.C.A. (1975) Morphine withdrawal response and central cholinergic activity. Nature 257, 131-132
Frederickson, R.C.A., Smits, S.E. (1973) Time course of dependence and tolerance development in rats treated with 'slow release' morphine suspensions. Res. Commun. Chem. Path. Pharmacol. 5, 867-870
Freed, W.J., Michaelis, E.K. (1976) Effects of intracisternal GABA or glutamic acid upon behavioural activity in the rat. Pharmacol. Biochem. Behav. 5, 11-14

Gale, K., Iadarola, M.J. (1980) Seizure protection and increased nerve-terminal GABA: delayed effects of GABA-transaminase inhibition. Science 208, 288-291
Garfinkel, D. (1966) A simulation study of the metabolism and compartmentation in brain of glutamate, aspartate, the Krebs cycle and related metabolites. J. Biol. Chem. 241, 3918-3929
Gellert, V.F., Holtzman, S.G. (1978) Development and maintenance of morphine tolerance and dependence in the rat by scheduled access to morphine drinking solutions. J. Pharmacol. Exp. Ther. 205, 536-546
Gent, J.P., Phillips, N.I. (1980) Sodium di-n-propylacetate (valproate) potentiates responses to GABA and muscimol on single central neurons. Brain Research 197, 275-278
Gerber, J.C., Hare, T.A. (1980) GABA in peripheral tissues: presence and actions in endocrine pancreatic function. In: GABA-Neurotransmission (Lal, H. ed) Brain Res. Bull.5, Suppl. 2 pp. 341-346
Gerig, J.T., Kwock, L. (1979) Inhibition of bacterial glutamate decarboxylase by tricarboxylic acid cycle intermediates. FEBS Lett. 105, 155-157
Gibson, A.C. (1977) Sodium valproate and tardive dyskinesia. Br. J. Psychiat. 133, 82.
Gispen, W.H., Wiegant, V.M., Bradbury, A.F., Hulme, E.C., Smyth, D.G., Snell, C.R., De Wied, D. (1976) Induction of excessive grooming in the rat by fragments of lipotropin. Nature 276, 794-795
Glick, S.D., Crane, L.A. (1978) Opiate-like and abstinence-like effects of intracerebral histamine administration in rats. Nature 273, 547-549
Godin, Y., Heiner, L., Mark, J., Mandel, P. (1969) Effects of di-n-propylacetate, an anticonvulsive compound, on GABA metabolism. J. Neurochem. 16, 869-873
Gruol, D.L., Barker, J.L., Smith, T.G. (1980) Naloxone antagonism of GABA-

evoked membrane polarizations in cultured mouse spinal cord neurons. Brain Research 198, 323-332
Gubler, C.J., Adams, B.L., Hammond, B., Yuan, E.C., Guo, S.M., Bennion, M. (1974) Effect of thiamine deprivation and thiamine antagonists on the level of γ-aminobutyric acid and on 2-oxoglutarate metabolism in rat brain. J. Neurochem. 22, 831-836

Harvey, P.K.P., Bradford, H.F., Davison, A.N. (1975) The inhibitory of sodium n-dipropylacetate on the degradative enzymes of the GABA shunt. FEBS Lett. 52, 251-254
Hayashi, T. (1959) The inhibitory action of β-hydroxy-γ-aminobutyric acid upon the seizure following stimulation of the motor cortex of the dog. J. Physiol. (London) 145, 570-578
Hayashi, T., Negishi, K. (1979) Suppression of retinal spike discharge by dipropylacetate (Depakene): a possible involvement of GABA. Brain Research 175, 271-278
Henriksen, S.J., Bloom, F.E., McCoy, F., Ling, N., Guillemin, R. (1978) β-Endorphin induces non-convulsive lumbic seizures. Proc. Nat. Acad. Sci. U.S.A. 75, 5221-5225
Hertz, L. (1979) Functional interactions between neurons and astrocytes. I. Turnover and metabolism of putative amino acid transmitters. Prog. Neurobiol. 13, 277-323
Ho, I.K., Gilliland,T.(1979) Effects of acute and continuous morphine administration on the affinity of glutamic acid decarboxylase for pyridoxal 5'-phosphate. Biochem. Pharmacol. 28, 355-360
Holtz, P., Palm, D. (1964) Pharmacological aspects of Vitamin B6. Pharmacol. Reviews 16, 113-178
Horton, R.W., Collins, J.F., Anlezark, G.M., Meldrum, B.S. (1979) Convulsant and anticonvulsant actions in DBA/2 mice of compounds blocking the reuptake of GABA. Europ. J. Pharmacol. 59, 75-83

Iadarola, M.J., Gale, K. (1979) Dissociation between drug-induced increases in nerve terminal and non-nerve terminal pools of GABA *in vivo*. Europ. J. Pharmacol. 59, 125-129
Iadarola, M.J., Raines, A., Gale, K. (1979) Differential effects of di-n-propylacetate and amino-oxyacetic acid on γ-aminobutyric acid levels in discrete areas of rat brain. J. Neurochem. 33, 1119-1123
Iversen, L.L. (1971) Role of transmitter uptake mechanisms in synaptic neurotransmission. Br. J. Pharmacol. 41, 571-591
Iversen, L.L., Kelly, J.S. (1975) Uptake and metabolism of γ-aminobutyric acid by neurones and glial cells. Biochem. Pharmacol. 24, 933-938
Iwatsubo, K., Kondo, Y. (1978) Inhibitory effect of morphine on the release of preloaded ^{3}H-GABA from rat substantia nigra in response to the stimulation of caudate nucleus and globus pallidus. In: Characteristics and function of opioids (Van Ree, J.M., Terenius, L., eds) Elsevier/ North Holland, Amsterdam pp. 357-358

Jackson, I.M.D., Reichlin, S. (1974) Thyrotropin-releasing hormone (TRH): distribution in hypothalamic and extrahypothalamic brain tissues of mammalian and submammalian chordates. Endocrinology, 95, 854-862
Jacquet, Y.F. (1978) Opiate effects after adrenocorticotropin or β-endorphin injection in the periaqueductal grey matter of rats. Science 201, 1032-1034
Jaffe, J.H. (1980) Drug addiction and drug abuse. In: The pharmacological basis of therapeutics. (Gilman, A.G., Goodman, L.S., Gilman, A., eds) MacMillan, New York pp. 535-584

Jaffe, J.H., Sharpless, S.K. (1968) Pharmacological denervation supersensitivity in the central nervous system: A theory of physical dependence. In: The addictive states. (Wikler, A. ed) Williams and Wilkins Comp., Baltimore, pp. 226-246

Jahn, U., Mixich, G. (1976) Wet dog shake behaviour in normal rats, elicited by benzylideneaminooxycarboxic acid derivatives. Psychopharmacologia 46, 191-196

John, R.A., Fowler, L.J. (1976) Kinetic and spectral properties of rabbit brain 4-aminobutyrate aminotransferase. Biochem. J. 155, 645-651

Kalivas, P.W., Horita, A. (1980) Thyrotropin-releasing hormone: neurogenesis of actions in the pentobarbital narcotized rat. J. Pharmacol. Exp. Ther. 212, 203-210

Kammeraat, C., Veldstra, H. (1967) Characterization of succinate semialdehyde dehydrogenase from rat brain. Biochim. Biophys. Acta 151, 1-10

Katz, R.I., Chase, T.N., Kopin, I.J. (1969) Effects of ions on stimulus induced release of amino acids from mammalian brain slices. J. Neurochem. 16, 961-967

Kerwin, R.W., Olpe, H.-R. (1980) The effect of sodium valproate on single unit activity in the rat brain. Br. J. Pharmacol. 70, 76 P.

Kerwin, R.W., Olpe, H.-R., Schmutz, M. (1980) The effect of sodium di-n-propylacetate on γ-aminobutyric acid-dependent inhibition in the rat cortex and substantia nigra in relation to its anticonvulsant activity. Br. J. Pharmacol. 71, 545-551

Kobayashi, K., Miyazawa, S., Endo, A. (1977) Isolation and inhibitory activity of gabaculine, a new potent inhibitor of γ-aminobutyrate aminotransferase produced by a streptomyces. FEBS Lett. 76, 207-210

Koob, G.F., Del Fiacco, M., Iversen, S.D. (1978) Spontaneous and amphetamine-induced behavior after bilateral injection of ethanol-O-sulphate into the substantia nigra. Brain Research 146, 313-323

Krnjevic, K. (1970) Glutamate and γ-aminobutyric acid in brain. Nature 228, 119-224

Kukino, K., Deguchi, T. (1977) Effects of sodium dipropylacetate on γ-aminobutyric acid and biogenic amines in rat brain. Chem. Pharmac. Bull. 25, 2257-2262

Kuriyama, K. (1976) Subcellular localization of the GABA system in brain. In: GABA in nervous system function. (Roberts, E., Chase, T.N., Tower, D.B., eds) Raven, New York pp. 187-196

Kuriyama, K., Roberts, E., Kakefuda, T. (1968 a) Association of the γ-aminobutyric acid system with a synaptic vesicle fraction from mouse brain. Brain Research 8, 132-152

Kuriyama, K., Roberts, E., Rubinstein, M.K. (1966) Elevation of γ-aminobutyric acid in brain with amino-oxyacetic acid and susceptibility to convulsive seizures in mice: a quantitative re-evaluation. J. Neurochem. 15, 221-236

Kuriyama, K., Roberts, E., Vos, J. (1968 b) Some characteristics of binding of γ-aminobutyric acid and acetylcholine to a synaptic vesicle fraction from mouse brain. Brain Research 9, 231-252

Kuschinsky, K., Bosse, A., Nöring, A., Ulmar, G. (1976) GABA and dopamine metabolism in the nigro-striatal pathways of rats. Alterations after single administrations of narcotics and during withdrawal. In: Opiates and endogenous opioid peptides. (Kosterlitz, H.W. ed) Elsevier/North Holland, Amsterdam pp. 419-422

LaBella, F.S., Havlicek, V., Pinsky, C. (1979 a) Opiate-like excitatory effects of steroid sulfates and calcium-complexing agents given cerebroventricularly. Brain Research 160, 295-305
LaBella, F.S., Havlicek, V., Pinsky, C., Leybin, L. (1978) Opiate-like, naloxone reversible effects of androsterone sulfate in rats. Can. J. Physiol. Pharmacol. 56, 940-944
LaBella, F.S., Pinsky, C., Havlicek, V. (1976 b) Morphine-derivatives with diminished opiate receptor potency show enhanced central excitatory activity. Brain Research 174, 263-271
Lacolle, J.Y., Ferrandes, B., Eymard, P. (1978) Profile of anticonvulsant activity of sodium valproate. Role of GABA. In: Advances in Epileptology-1977. (Meinardi, H., Rowan, A.J., eds) Swets and Zeitlinger, Amsterdam pp. 162-167
Lagowska, J., Calvino, B., Ben-Ari, Y. (1978) Intra-amygdaloid application of naloxone elicits severe withdrawal signs in morphine-dependent rats. Neurosci. Lett. 8, 241-245
Lal, H., Colpaert, F.C., Laduron, P.(1975) Narcotic withdrawal-like mouse-jumping produced by amphetamine and L-DOPA. Europ. J. Pharmacol. 30, 113-116
Lanthorn, T., Isaacson, R.L. (1978) Studies on kainate induced wet-dog shakes in the rat. Life Sciences 22, 171-178
Lanthorn, T., Smith, M.A., Isaacson, R.L. (1979) Wet-dog shaking in the rat: possible involvement of a kappa opiate receptor. Neuropharmacology 18, 743-745
Laschka, E., Herz, A. (1977) Sites of action of morphine involved in the development of physical dependence in rats III. Autoradiographic studies. Psychopharmacology 53, 33-37
Laschka, E., Teschemacher, Hj., Mehraein, P., Herz, A. (1976) Sites of action of morphine involved in the development of physical dependence in rats II. Morphine withdrawal precipitated by application of morphine antagonists into restricted parts of the ventricular system and by microinjection into various brain areas. Psychopharmacologia 46, 141-147
Laverty, R., Roth, R.H. (1980) Clonidine reverses the increased norepinephrine turnover during morphine withdrawal in rats. Brain Research 182, 482-485
Levi, G., Raiteri, M. (1978) Modulation of γ-aminobutyric acid transport in nerve endings: role of extracellular γ-aminobutyric acid and of cationic fluxes. Proc. Nat. Acad. Sci. U.S.A. 75, 2981-2985
Leybin, L., Pinsky, C., LaBella, F.S., Havlicek, V., Rezek, M. (1976) Intraventricular Met^5-enkephalin causes unexpected lowering of pain threshold and narcotic withdrawal signs in rats. Nature 264, 458-459
Lin, S.C., Sutherland, V.C., Way, E.L. (1973) Brain amino acids in morphine tolerant and non-tolerant rats. Proc. West. Pharmacol. Soc. 16, 8-13
Llorens, C., Martres, M.P., Baudry, M., Schwartz, J.C. (1978) Hyper-sensitivity to noradrenaline in cortex after chronic morphine: relevance to tolerance and dependence. Nature 274, 603-605
Lust, W.D., Kupferberg, H.J., Yonekawa, W.D., Penry, J.K., Passoneau, J.V., Wheaton, A.B. (1978) Changes in brain metabolites induced by convulsants or electroshock: effects of anticonvulsant agents. Molec. Pharmacol. 14, 347-356

MacDonald, R.L., Bergey, G.K. (1979) Valproic acid augments GABA-mediated postsynaptic inhibition in cultured mammalian neurons. Brain Research 170, 558-562
Maitre, M., Cieselski, L., Cash, C., Mandel, P. (1975) Purification and studies on some properties of the 4-aminobutyrate:2-oxoglutarate trans-aminase from rat brain. Europ. J. Biochem. 52, 157-169

Maitre, M., Cieselski, L., Cash, C., Mandel, P. (1978 a) Comparison of the structural characteristics of the 4-aminobutyrate:2-oxoglutarate transaminases from rat and human brain, and their affinities for certain inhibitors. Biochem. Biophys. Acta 522, 385-399
Maitre, M., Blindermann, J.M., Ossola, L., Mandel, P. (1978 b) Comparison of the structures of L-glutamate decarboxylases from human and rat brains. Biochem. Biophys. Res. Commun. 85, 885-890
Mangan, J.L., Whittaker, V.P. (1966) The distribution of free amino acids in subcellular fractions of guinea-pig brain. Biochem. J. 98, 128-137
Martin, B.R., Dewey, W.L., Chau-Pam, T., Prange, A.J. (1977) Interactions of thyrotropin-releasing hormone and morphine sulfate in rodents. Life Sciences 20, 715-722
Martin, W.R., Wikler, A., Eades, C.G., Pescor, F.T. (1963) Tolerance to and physical dependence on morphine in rats. Psychopharmacologia 4, 247-260
Matsuda, M., Abe, M., Hoshino, M., Sakurai, T. (1979) γ-Aminobutyric acid in subcellular fractions in mouse brain and its relation to convulsions. Biochem. Pharmacol. 28, 2785-2789
Matsuda, M., Hoshino, M., Sakurai, T. (1978) Synaptosomal γ-aminobutyric acid and glutamic acid contents and its relationship to convulsions. Jikeikai Med. J. 25, 87-93
Matsui, Y., Deguchi, T. (1977) Effects of gabaculine, a new potent inhibitor of gamma-aminobutyrate transaminase, on the brain gamma-aminobutyrate content and convulsions in mice. Life Sciences 20, 1291-1296
Matsui, Y., Kamioka, T. (1978 a) The effects of elevating γ-aminobutyrate content in the substantia nigra on the behaviour of rats. Europ. J. Pharmacol. 50, 243-251
Matsui, Y., Kamioka, T. (1978 b) Cataleptic and anticataleptic effects of muscimol and gabaculine injected into the globus pallidus and substantia nigra and interactions with haloperidol or benzodiazepines. Naunyn - Schmied. Arch. Pharmacol. 305, 219-225
Matsui, Y., Kamioka, T. (1979) Effects of intracerebral injection with muscimol on locomotor activity and catalepsy in rats. Japan. J. Pharmacol. 29, 496-499
Maynert, E.W., Klingman, G.I., Kaji, H.K. (1962) Tolerance to morphine II. Lack of effects on brain 5-hydroxytryptamine and γ-aminobutyric acid. J. Pharmacol. Exp. Ther. 135, 296-299
McGeer, P.L., McGeer, E.G., Wada, J.A., Jung, E. (1971) Effects of globus pallidus lesions and Parkinson's disease on brain glutamic acid decarboxylase. Brain Research 32, 425-431
Meldrum, B.S. (1975) Epilepsy and γ-aminobutyric acid mediated inhibition. Int. Rev. Neurobiol. 17, 1-36
Meldrum, B. (1978) Convulsant drugs, anticonvulsants and GABA-mediated neuronal inhibition. In: GABA-Neurotransmitters. Pharmacochemical, Biochemical and Pharmacological aspects (Krogsgaard-Larsen, P., Scheel-Krüger, J., Kofod, H., eds) Munksgaard, Copenhagen, pp. 390-405
Metcalf, B.W. (1979) Inhibitions of GABA metabolism. Biochem. Pharmacol. 28, 1705-1712
Miller, L.P., Martin, D.L., Walters, J.R. (1977) *Post-mortem* changes implicate adenine nucleotides and pyridoxal-5'-phosphate in regulation of brain glutamate decarboxylase. Nature 266, 847-848
Miller, A.L., Pitts, F.N. (1967) Brain succinate semialdehyde dehydrogenase-III Activities in twenty-four regions of human brain. J. Neurochem. 14, 579-584
Mitchell, P.R., Martin, I.L. (1978) Is GABA release modulated by presynaptic receptors? Nature 274, 904-905

Mogenson, G.J., Wu, M., Manchanda, S.K. (1979) Locomotor activity initiated by micro infusions of picrotoxin into the ventral tegmental area. Brain Research 161, 311-319
Moroni, F., Cheney, D.L., Peralta, E., Costa, E. (1978) Opiate receptor agonists as modulators of γ-aminobutyric acid turnover in the nucleus caudatus, globus pallidus and substantia nigra of the rat. J. Pharmacol. Exp. Ther. 207, 870-877
Moroni, F., Peralta, E., Cheney, D.L., Costa, E. (1979) On the regulation of γ-aminobutyric acid neurons in caudatus, pallidus and nigra: Effects of opioids and dopamine agonists. J. Pharmacol. Exp. Ther. 208, 190-194

Ngo, T.T., Tunnicliff, G. (1978) Further evidence for the existence of isozymes of brain γ-aminobutyrate aminotransferase. Comp. Biochem. Physiol. 59 C, 101-104
Nistri, A., Constanti, A. (1979) Pharmacological characterization of different types of GABA and glutamate receptors in vertebrates and invertebrates. Prog. Neurobiol. 13, 117-235
Nitsch, C. (1980) Regulation of GABA metabolism in discrete rabbit brain regions under methoxy pyridoxine - Regional differences in cofactor saturation and the preictal activation of glutamate decarboxylase activity. J. Neurochem. 34, 822-830

Obata, K., Takeda, K. (1969) Release of GABA into the fourth ventricle induced by stimulation of the cat cerebellum. J. Neurochem. 16, 1043-1047
Ostrovskaya, R.U., Molodavkin, G.M. (1976) The GABA-ergic mechanism of the effect of diazepam on cortical neurons. Byull. Eksp. Biol. Med. 82, 1073-1076
Otsuki, S., Watanabe, S., Ninomiya, K., Hoaki, T., Okomura, N. (1968) Correlation between [U-^{14}C] glucose metabolism and function in perfused cat brain. J. Neurochem. 15, 859-865

Pérez de la Mora, M., Feria-Velasco, A., Tapia, R. (1973) Pyridoxal-phosphate and glutamate decarboxylase in subcellular particles of mouse brain and their relationship to convulsions. J. Neurochem. 20, 1575-1587
Perry, T.L., Hansen, S., Kloster, M. (1973) Huntington's chorea, deficiency of γ-aminobutyric acid in brain. New Engl.J. Medic. 288, 337-342
Pert, A. (1978) Central sites involved in opiate actions. In: The bases of addiction. (Fishman, J., ed) Dahlem Konferenzen, Berlin pp. 299-332
Prange, A.J., Breese, G.R., Cott, J.M., Martin, B.R., Cooper, B.R., Wilson, I.C., Plotnikoff, N.P. (1974) Thyrotropin-release hormone: antagonism of pentobarbital in rodents. Life Sciences 14, 447-455
Przewlocka, B., Stala, L., Scheel-Krüger, J. (1979) Evidence that GABA in the nucleus dorsalis raphé induces stimulation of locomotor activity and eating behavior. Life Sciences 25, 937-946

Reubi, J.-C., Van den Berg, C., Cuénod, M. (1978) Glutamine as precursor for the GABA and glutamate transmitter pools. Neurosci. Lett. 10, 171-174
Roberts, E. (1972) An hypothesis suggesting that there is a deficit in the GABA system in schizophrenia. Neurosci. Res. Program. Bull. 10, 468-481
Roberts, E. (1980) Epilepsy and antiepileptic drugs: a speculative synthesis. In: Antiepileptic drugs: Mechanism of action. (Glaser, G.H., Penry, J.K, Woodbury, D.M., eds) Adv. Neurology Vol. 27, Raven, New York pp. 667-713
Roberts, E., Frankel, S. (1950) γ-Aminobutyric acid in brain: its formation from glutamic acid. J. Biol. Chem. 187, 55-63
Roberts, E., Rothstein, M., Baxter, C.F. (1958) Some metabolic studies of

γ-aminobutyric acid. Proc. Soc. Exp. Biol. Med. 97, 796-802
Rosenfeld, G.C., Strada, S.J., Robison, G.A. (1979) Cyclic nucleotides and the central effects of opiates: An overview of current research. In:Neurochemical mechanisms of opiates and endorphins. Adv. Biochem. Psychopharmacol. Vol. 20 (Loh, H.H., Ross, D.H. eds) Raven, New York pp. 261-280
Ross, D.H., Cardenas, H.L. (1979) Nerve cell calcium as a messenger for opiate and endorphin actions. In: Neurochemical mechanisms of opiates and endorphins. Adv. Biochem. Psychopharmacol. Vol. 20 (Loh, H.H., Ross, D.H., eds) Raven, New York pp. 301-336
Ryan, L.D., Roskoski, R. (1977) Net uptake of γ-aminobutyric acid by a high affinity synaptosomal transport system. J. Pharmacol. Exp. Ther. 200, 285-291

Salganicoff, L., DeRobertis, E. (1963) Subcellular distribution of glutamic decarboxylase and gamma-aminobutyric alpha-ketoglutaric transaminase. Life Sciences 2, 85-91
Salganicoff, L., DeRobertis, E. (1965) Subcellular distribution of the enzymes of the glutamic acid, glutamine and γ-aminobutyric acid cycles in rat brain. J. Neurochem. 12, 287-309
Sano, K., Roberts, E. (1961) Binding of γ-aminobutyric acid by brain preparations. Biochem. Biophys. Res. Commun. 4, 358-361
Sarhan, S., Seiler, N. (1979) Metabolic inhibitors and subcellular distribution of GABA. J. Neurosci. Res. 4, 399-421
Sawaya, M.C.B., Horton, R.W., Meldrum, B.S. (1975) Effects of anticonvulsant drugs on the cerebral enzymes metabolizing GABA. Epilepsia 16, 649-655
Schechter, P.J., Tranier, Y., Grove, J. (1979) Gabaculine and isogabaculine: *in vivo* biochemistry and pharmacology in mice. Life Sciences 24, 1173-1182
Schechter, P.J., Tranier, Y., Jung, M.J., Böhlen, P. (1977) Audiogenic seizure protection by elevated brain GABA concentration in mice: effects of γ-acetylenic GABA and γ-vinyl GABA, two irreversible GABA-T inhibitors. Europ. J. Pharmacol. 45, 319-328
Scheel-Krüger, J., Arnt, J., Breastrup, C., Christensen, A.V., Cools, A.R., Magelund, G. (1978 a) GABA-dopamine interaction in substantia nigra and nucleus accumbens - relevance to behavioral stimulation and stereotyped behavior. In: Dopamine (Roberts, P.J., Woodruff, G.N., Iversen, L.L.,eds) Adv. Biochem. Psychopharmacol. Vol. 19, Raven, New York pp. 343-346
Scheel-Krüger, J., Christensen, A.V., Arnt, J. (1978 b) Muscimol differentially facilitates stereotypy but antagonizes motility induced by dopaminergic drugs: a complex GABA-dopamine interaction. Life Sciences 22, 75-84
Scheel-Krüger, J., Cools, A.R., Van Wel, P. (1977) Muscimol, a GABA-agonist injected into the nucleus accumbens increases apomorphine stereotypy and decreases the motility. Life Sciences 21, 1697-1702
Schenkel-Hulliger, L., Koella, W.P., Hartmann, A., Maitre, L. (1974) Tremorogenic effect of thyrotropin-releasing hormone in rats. Experienta 30, 4-6
Schmidt, W.K., Way, E.L. (1980) Effect of a calcium chelator on morphine tolerance development. Europ. J. Pharmacol. 63, 243-250
Schmutz, M., Olpe, H.-R., Koella, W.P. (1979) Central actions of valproate sodium. J. Pharm. Pharmacol. 31, 413-414
Schneider, C. (1968) Behavioural effects of some morphine antagonists and hallucinogens in the rat. Nature 220, 586-587
Schousboe, A. (1978) Significance of the glial transport system for the inactivation of GABA and effect of structural analogues on the uptake.

In: GABA-Neurotransmitters. Pharmacochemical, Biochemical and Pharmacological aspects. (Krogsgaard-Larsen, P., Scheel-Krüger, J., Kofod, H., eds) Munksgaard, Copenhagen pp. 263-280
Schousboe, A., Wu, J.-Y., Roberts, E. (1973) Purification and characterization of the 4-aminobutyrate-2-ketoglutarate transaminase from mouse brain. Biochemistry, Easton 12, 2868-2873
Schousboe, A., Wu, J.-Y., Roberts, E. (1974) Subunit structure and kinetic properties of 4-aminobutyrate-2-ketoglutarate transaminase purified from mouse brain. J. Neurochem. 23, 1189-1195
Scotti de Carolis, A., Massotti, M. (1978) Electroencephalographic and behavioral investigations on "GABA-ergic"drugs: muscimol, baclofen and sodium γ-hydroxybutyrate. Prog. Neuro-Psychopharmacol. 2, 431-442
Segal, M. (1977) Morphine and enkephalin interactions with putative neurotransmitters in rat hippocampus. Neuropharmacology 16, 587-592
Seiler, N., Sarhan, S. (1980) Drugs affecting GABA. In: Neurochemistry and Clinical Neurology, in press.
Sethy, V.H., Bombardt, P.A. (1978) Is GABA involved in analgesia? Res. Commun. Chem. Path. Pharmac. 19, 365-368
Shahid Salles, K.S., Colasanti, B.K., Craig, C.R. (1979) Cyclic AMP-induced changes in the electroencephalogram and in behavior during morphine dependence and abstinence in the rat. Sleep 1, 247-257
Sheridan, J.J., Sims, K.L., Pitts, F.N. (1967) Brain γ-aminobutyrate-α-oxoglutarate transaminase - II. Activities in twenty-four regions of human brain. J. Neurochem. 14, 571-578
Simler, S., Cieselski, L., Maitre, M., Randrianarisoa, H., Mandel, P. (1973) Effect of sodium n-dipropylacetate on audiogenic seizures and brain γ -aminobutyric acid level. Biochem. Pharmacol. 22, 1701-1708
Simler, S., Gensburger, C., Cieselski, L., Mandel, P. (1978) Time course of the increase in GABA level in different mice brain regions following n-dipropylacetate treatment. Commun. Psychopharmacol. 2, 123-130
Sims, K.L., Davis, G.A. (1973) Subcellular distribution of succinate semialdehyde dehydrogenase in rat brain. Europ. J. Biochem. 35, 450-453
Sloviter, R.S., Drust, E.G., Connor, J.D. (1978) Specificity of a rat behavioral model for serotonin receptor activation. J. Pharmacol. Exp. Ther. 206, 339-347
Srinivasan, V., Neal, M.J., Mitchell, J.F. (1969) The effect of electrical stimulation and high potassium concentrations on the efflux of ^{3}H γ-aminobutyric acid from brain slices. J. Neurochem. 16, 1235-1244
Storm-Mathisen, J. (1976) Distribution of the components of the GABA system in neuronal tissue: Cerebellum and hippocampus - effects of axotomy. In: GABA in nervous system function. (Roberts, E., Chase, T.N., Tower, D.B., eds) Raven, New York pp. 149-168
Sytinski, I.A., Vasilijev, V.Y. (1969) Some catalytic properties of purified γ-aminobutyrate-α-oxoglutarate transaminase from the rat brain. Enzymologia 39, 1-11

Taberner, P.V., Roberts, F. (1978) The anticonvulsant action of L-2,4-diaminobutyric acid. Europ. J. Pharmacol. 52, 281-286
Takanaka, A., Tzeng, S.F., Ho, I.K. (1976) Acute and chronic effects of morphine on glutamic acid decarboxylase (GAD) activity and γ-aminobutyric acid (GABA) level in discrete brain areas of the mouse. Neurosci. Abstr. 2, 617
Tanner, T. (1979) GABA-induced locomotor activity in the rat after bilateral injection into the ventral tegmental area. Neuropharmacology 18, 441-446
Tapia, R., Gonzalez, R.M. (1978) Glutamine and glutamate as precursors of the releasable pool of GABA in brain cortex slices. Neurosci. Lett. 10, 165-169

Tapia, R., Sandoval, M.E., Contreras, P. (1975) Evidence for a role of glutamate decarboxylase activity as a regulatory mechanism of cerebral excitability. J. Neurochem. 24, 1283-1285

Tappaz, M.L., Brownstein, M.J., Kopin, I.J. (1977) Glutamate decarboxylase (GAD) and γ-aminobutyric acid (GABA) in discrete nuclei of hypothalamus and substantia nigra. Brain Research 125, 109-121

Toffano, G., Guidotti, A., Costa, E. (1978) Purification of an endogenous protein inhibitor of the high affinity binding of gamma-aminobutyric acid to synaptic membranes of rat brain. Proc. Nat. Acad. Sci. U.S.A. 75, 4024-4028

Toffano, G., Leon, A., Massotti, M., Guidotti, A., Costa, E. (1980) GABA-modulin: a regulatory protein for GABA receptors. In: Receptors for neurotransmitters and peptide hormones. (Pepeu, G., Kuhar, M.J., Enna, S.J., eds) Adv. Biochem. Psychopharmacol. Vol. 21 Raven, New York pp. 133-142

Tower, D.B. (1976) GABA and seizures: clinical correlates in man. In: GABA in nervous sytem function. (Roberts, E., Chase, T.N., Tower, D.B., eds) Raven, New York pp. 461-478

Tzeng, S.F., Ho, I.K. (1978) Acute and continuous morphine administration on the γ-aminobutyric acid system in the mouse. Prog. Neuro-Psychopharmacol. 2, 55-64

Udenfriend, S. (1950) Identification of γ-aminobutyric acid in brain by the isotope derivative method. J. Biol. Chem. 187, 65-69

Van den Berg, C.J., Garfinkel, D. (1971) A simulation study of brain compartments. Biochem. J. 123, 211-218

Van den Berg, C.J., Matheson, D.F., Ronda, G., Reynierse, G.L.A., Blokhuis, G.G.D., Kroon, M.C., Clarke, D.D., Garfinkel, D. (1975) A model of glutamate metabolism in brain: A biochemical analysis of heterogenous structure. In: Metabolic compartmentation and Neurotransmission. (Berl, S., Clarke, D.D., Schneider, D., eds) Plenum, New York pp. 515-543

Van der Heyden, J.A.M., De Kloet, E.R., Korf, J., Versteeg, D.H.G. (1979 b) GABA content of discrete brain nuclei and spinal cord of the rat. J. Neurochem. 33, 857-861

Van der Heyden, J.A.M., Korf, J. (1978) Regional levels of GABA in the brain: rapid semiautomated assay and prevention of *post mortem* increase by 3-mercaptopropionic acid. J. Neurochem. 31, 197-203

Van der Heyden, J.A.M., Venema, K., Korf, J. (1979 a) *In vivo* release of endogenous GABA from rat substantia nigra measured by a novel method. J. Neurochem. 32, 469-476

Van der Heyden, J.A.M., Venema, K., Korf, J. (1980) *In vivo* release of endogenous GABA from rat striatum: effects of muscimol, oxotremorine and morphine. J. Neurochem. 34, 1648-1653

Van Kammen, D.P. (1977) γ-Aminobutyric acid (GABA) and the dopamine hypothesis of schizophrenia. Am. J. Psychiat. 134, 138-143

Van Kempen, G.M.J., Van den Berg, C.J., Van der Helm, H.J., Veldstra, H. (1965) Intracellular localization of glutamate decarboxylase, γ-amino-butyrate transaminase and some other enzymes in brain tissue. J. Neurochem. 12, 581-588

Volicer, L., Puri, S.K., Choma, P. (1977) Cyclic GMP and GABA levels in rat striatum and cerebellum during morphine withdrawal: effect of apo-morphine. Neuropharmacology 16, 791-794

Von Voigtlander, P.F., Losey, E.G. (1977) Prostaglandin E_2, cyclic adenosine monophosphate and morphine analgesia. Brain Research 128, 275-283

Wachtel, H., Andén, N.-E. (1978) Motor activity of rats following intracerebral injections of drugs influencing GABA mechanisms. Naunyn-Schmied. Arch. Pharmacol. 302, 133-139
Waksman, A., Bloch, M. (1968) Identification of multiple forms of γ-aminobutyrate transaminase in mouse and rat brain: subcellular localization. J. Neurochem. 15, 99-105
Waksman, A., Roberts, E (1965) Purification and some properties of mouse brain γ-aminobutyrate-α-ketoglutaric acid transaminase. Biochemistry, Easton 4, 2132-2139
Waksman, A., Rubinstein, M.K., Kuriyama, K., Roberts, E. (1968) Localization of γ-aminobutyric-α-oxoglutaric acid transaminase in mouse brain. J. Neurochem. 15, 351-357
Wallach, D.P. (1961) Studies on the GABA pathway. I: The inhibition of γ-aminobutyric acid-α-ketoglutaric acid transaminase *in vitro* and *in vivo* by U-7524 (amino-oxyacetic acid).Biochem. Pharmacol. 5, 323-331
Walsh, J.M., Clark, J.B. (1976) Studies on the control of 4-aminobutyrate metabolism in "synaptosomal" and free rat brain mitochondria. Biochem. J. 160, 147-157
Way, E.L. (1978) Common and selective mechanisms in drug dependence. In: The bases of addiction. (Fishman, J., ed) Dahlem Konferenzen, Berlin pp. 333-352
Way, E.L., Loh, H.H., Shen, F.H. (1969) Simultaneous quantitative assessment of morphine tolerance and physical dependence. J. Pharmacol. Exp. Ther. 167, 1-8
Wei, E.T. (1976) Chemical stimulation of shaking behaviour. J. Pharm. Pharmacol. 28, 722-724
Wei, E.T., Loh, H.H., Way, E.L. (1972) Neuroanatomical correlates of morphine dependence. Science 177, 616-617
Wei, E., Loh, H.H., Way, E.L. (1973 a) Quantitative aspects of precipitated abstinence in morphine-dependent rats. J. Pharmacol. Exp. Ther. 184, 398-403
Wei, E., Loh, H.H., Way, E.L. (1973 b) Brain sites of precipitated abstinence in morphine-dependent rats. J. Pharmacol. Exp. Ther. 185, 108-115
Wei, E., Sigel, S., Loh, H.H., Way, E.L. (1975 a) Thyrotropin-releasing hormone and shaking behaviour in rat. Nature 253, 739-740
Wei, E., Sigel, S.S.R., Loh, H.H., Way, E.L. (1975 c) Central sites of naloxon-precipitated shaking in the anesthetized morphine-dependent rat. J. Pharmacol. Exp. Ther. 195, 480-487
Wei, E., Sigel, S., Way, E.L. (1975 b) Regional sensitivity of the rat brain to the inhibitory effects on morphine on wet shake behavior. J. Pharmacol. Exp. Ther. 193, 56-63
Whelan, D.T., Scriver, C.R., Mohyuddin, F. (1969) Glutamic acid decarboxylase and gamma-aminobutyric acid in mammalian kidney. Nature 224, 916-917
Winokur, A., Utiger, R.D. (1974) Thyrotropin-releasing hormone: regional distribution in rat brain. Science 185, 265-267
Wood, J.D. (1975) The role of gamma-aminobutyric acid in the mechanism of seizures. Prog. Neurobiol. 5, 77-95
Wood, J.D., Durham, J.S., Peesker, S.J. (1977) Effect of di-n-propylacetate and γ-acetylenic GABA on hyperbaric oxygen-induced seizures and GABA metabolism. Neurochem. Res. 2, 707-715
Wood, J.G., McLaughlin, B.J., Vaughn, J.E. (1976) Immunocytochemical localization of GAD in electronmicroscopic preparations of rodent CNS. In: GABA in nervous system function. (Roberts, E., Chase, T.N., Tower, D.B., eds) Raven, New York pp. 133-148
Wood, J.D., Peesker, S.J. (1973) The role of GABA metabolism on the convulsant and anticonvulsant actions of aminooxyacetic acid.

J. Neurochem. 20, 379-387
Wood, J.D., Peesker, S.J. (1974) Development of an expression which relates the excitable state of the brain to the level of GAD activity and GABA content, with particular reference to the action of hydrazine and its derivatives. J. Neurochem. 23, 703-712
Wood, J.D., Peesker, S.J. (1975) The anticonvulsant action of GABA-elevating agents: a re-evaluation. J. Neurochem. 25, 277-282
Wood, J.D., Russell, M.P., Kurylo, E. (1980) The γ-aminobutyrate content of nerve endings (synaptosomes) in mice after the intramuscular injection of γ-aminobutyrate-elevating agents: a possible role in anticonvulsant activity. J. Neurochem. 35, 125-130
Wood, J.D., Russell, M.P., Kurylo, E., Newstead, J.D. (1979) Stability of synaptosomal GABA levels and their use in determining the *in vivo* effects of drugs: convulsant agents. J. Neurochem. 33, 61-68
Worms, P., Lloyd, K.G. (1980) Biphasic effects of direct, but not indirect, GABA mimetics and antagonists on haloperidol-induced catalepsy. Naunyn-Schmied. Arch. Pharmacol. 311, 179-184
Wu, J.-Y., Matsuda, T., Roberts, E. (1973) Purification and characterization of glutamate decarboxylase from mouse brain. J. Biol. Chem. 248, 3029-3034
Wu, J.-Y., Roberts, E. (1974) Properties of brain L-glutamate decarboxylase: inhibition studies. J. Neurochem. 23, 759-767

Zachmann, M., Tocci, P., Nyhan, W.L. (1966) The occurence of γ-aminobutyric acid in human tissues other than brain. J. Biol. Chem. 241, 1355-1358
Zisappel, N., Zurgil, N. (1978) The content of GABA and other amino acids in bovine cerebral cortex synaptic vesicles. Life Sciences 23, 231-236
Zukin, S.R., Young, A.B., Snyder, S.H. (1974) Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system. Proc. Nat. Acad. Sci. U.S.A. 71, 4802-4807

CHAPTER II

DI-n-PROPYLACETATE AND GABA DEGRADATION PREFERENTIAL INHIBITION OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE AND INDIRECT INHIBITION OF GABA-TRANSAMINASE

J.W. van der Laan, Th. de Boer and J. Bruinvels

Published in Journal of Neurochemistry 32 (1979) 1769-1780

ABSTRACT

The kinetic constants for 4-aminobutyrate: 2-oxoglutarate aminotransferase (GABA-transaminase) and succinate-semialdehyde: NAD^+ oxidoreductase (SSA-DH) have been determined using rat brain homogenate.

The Michaelis constants for GABA-T at saturated substrate concentrations were calculated to be K_{GABA} = 1.5 mM, $K_{2\text{-}OG}$ = 0.25 mM, K_{Glu} = 620 µM and K_{SSA} = 87 µM. The V_{max} for the reaction using GABA and 2-oxoglutarate (2-OG) as substrates (forward reaction) was found to be 35.2 µmol/g/h, whereas for the reaction using glutamate (Glu) and succinate-semialdehyde (SSA) a value of 63.0 µmol/g/h was found.

The kinetics of GABA-T have been shown to be consistent with a Ping Pong Bi Bi mechanism. Substrate inhibition of the forward reaction, through formation of a dead-end complex, was found to occur with 2-OG (K_i = 3.3 mM), whereas GABA was found to be a product inhibitor of the reverse reaction (K_i = 0.6 mM). Using the appropriate Haldane relationship, a K_{eq} of 0.04 for GABA-T was found, indicating that the reaction was strongly biased towards GABA.

For SSA-DH, the K_m of SSA was determined (9.1 µM) and the V_{max} was 27.5 µmol/g/h. The effect of di-n-propylacetate (DPA) on both GABA-T and SSA-DH was measured. DPA inhibited SSA-DH competitively with respect to SSA, giving a K_i of 0.5 mM. GABA-T was only slightly inhibited. The K_i of DPA for the forward reaction was 23.2 mM with respect to GABA, which was 40-50 times higher than that for SSA-DH. For the reverse reaction the K_i of DPA was found to be nearly the same (15.2 mM with respect to Glu and 22.9 mM with respect to SSA). These results suggest that GABA accumulation in the brain, after administration of DPA *in vivo*, is caused by SSA-DH inhibition. Two mechanisms are indicated by the data. (1) The higher level of SSA, which

results from inhibition of SSA-DH, initiates the reverse reaction of GABA-T, thus increasing the level of GABA via conversion of SSA. (2) The degradation of GABA is inhibited by SSA, since SSA has a strong inhibitory effect on the forward reaction, as calculated from the present data.

INTRODUCTION

In mammalian brain gamma-aminobutyric acid (GABA) is thought to be an inhibitory neurotransmitter (Krnjević, 1970). A decrease in the concentration of GABA has been implicated in the onset of convulsions (Tower, 1976). Accordingly, it has been reported by many authors that several anticonvulsant drugs increase GABA levels in the brain through inhibition of its degradation (see Tapia, 1975).

Elevation of GABA levels has been extensively studied by administration of drugs which affect the activity of 4-aminobutyrate: 2-oxoglutarate amino-transferase (EC 2.6.1.19; GABA-T). Such drugs include aminooxyacetic acid (AOAA; Baxter & Roberts, 1961), ethanolamine-O-sulphate (EOS; Fowler & John, 1972), γ-acetylene-GABA and γ-vinyl-GABA (Schechter et al., 1977; Jung et al., 1977). Similarly, di-n-propylacetate (Depakine®, Valproate, DPA) also increases the concentration of GABA in the brain (Anlezark et al., 1976; Godin et al., 1969; Simler et al., 1973) and its action has been ascribed to an inhibition of GABA-T (Godin et al., 1969; Simler et al., 1973; Fowler et al., 1975). However, some authors reported a preferential action of DPA on SSA-DH (Harvey et al., 1975; Anlezark et al., 1976). Two specific reactions are involved in the degradation of GABA; firstly, the reversible conversion of GABA and 2-oxoglutarate (2-OG) into succinate-semialdehyde (SSA) and glutamate (Glu), respectively, which is catalysed by GABA-T, and secondly, the subsequent oxidation of SSA to form succinate by succinate-semialdehyde: NAD^+ oxido-reductase (EC 1.2.1.16; SSA-DH) using NAD as a cofactor, which reaction is almost irreversible. Since the equilibrium of the GABA-T catalysed reaction is biased towards GABA (Duffy et al., 1972), inhibition of SSA-DH may also result in elevation of the GABA concentration.However, this possibility has so far been neglected, because SSA-DH was considered to be very efficient in preventing accumulation of SSA. Recent studies have suggested that the activity of SSA-DH can be affected by mono- and bivalent cations like sodium, potassium and calcium (De Boer & Bruinvels, 1977b). Therefore, it is possible that SSA-DH may play a regulating role in GABA degradation *in vivo* and that inhibition of this enzyme might increase GABA-concentrations through

activating the reverse reaction of GABA-T.

The aim of the present paper has, therefore, been to study this regulatory role of SSA-DH in relation to the action of DPA. The affinity of SSA for both GABA-T and SSA-DH and its alteration by DPA have been determined as well as the effect of DPA on the conversion of GABA into SSA.

MATERIALS AND METHODS

Materials

GABA was obtained from Calbiochem, Los Angeles, CA. Triton X-100, 2-oxo-glutaric acid, di-sodium succinate and 2-mercaptoethanol were obtained from BDH Chemicals Ltd. Pyridoxamine-di-HCl and SSA were purchased from Sigma Chemical Company. NAD and NADH were obtained from Boehringer-Mannheim. [$U^{14}C$] GABA (specific radioactivity 4.6 mCi/mmol) was obtained from the Radio-chemical Centre, Amersham, UK. All other chemicals were purchased from Merck, Darmstadt. DPA was a gift of Labaz B.V., Maassluis, The Netherlands.

Tissue preparation

Randomly selected male Wistar rats (125-175 g) were killed by decapitation and their brains were rapidly removed and weighed. A 10% (w/v) brain homogenate was prepared in an ice-cold solution containing 0.32 M-sucrose and 4.5 mM-2-mercaptoethanol, using a Teflon-glass Potter-Elvehjem homogenizer. One volume of the homogenate was added to 3 vol of ice-cold Triton medium (0.67% w/v Triton X-100, 50 mM-Tris-HCl (pH 8.5) and 4.5 mM-2-mercaptoethanol) and kept on ice-water for 1 h before use.

GABA-T assay using GABA as substrate (forward reaction)

For the performance of the assay of the GABA-T forward reaction a radio-chemical method was used as previously described (De Boer & Bruinvels, 1977a) with the following modifications. NAD was omitted from the incubation mixture, unless otherwise specified, to exclude interference with SSA-DH, since DPA might affect this enzyme too. A control was carried throughout the whole procedure in the presence of NAD but without DPA. Tissue concentration was 4.44 mg/ml (wet wt.). After preincubation at 22°C for 30 min the reaction was started by the addition of homogenate and the mixture was incubated for a further 30 min at the same bath temperature. All experiments were carried out in duplicate.

GABA-T assay using SSA as substrate (reverse reaction)

The reaction was carried out under similar conditions to the forward reaction. The incubation mixture contained 50 mM-Tris-HCl (pH 8.5), 20 mM

2-mercaptoethanol and 0.12 ml Triton-treated homogenate in a total volume of 0.615 ml. The final tissue concentration was 4.44 mg/ml. The reaction mixture was preincubated at 22°C for 1 h in the presence of glutamate (0.5-5.0 mM) and in the absence of SSA. The reaction was started by addition of 60 µl SSA solution, resulting in final concentrations of 50-600 µM-SSA. For the inhibition studies DPA was added simultaneously with SSA.

Since the sodium salt of DPA was used throughout this study, a solution in which DPA was replaced by sodiumisethionate, equimolar to the highest DPA concentration, was used as control.

After an incubation period of 15 or 20 min, the reaction was terminated by adding an equal volume of ice-cold 5% TCA solution. Subsequently, the tubes were placed in ice-water. After centrifugation (10 min x 1200 g) the concentration of 2-OG in 0.5 ml of the supernatant was determined in duplicate.

Determination of 2-oxoglutarate

The fluorometric determination of α-oxo acids as described by Takeda et al. (1976) was used. To neutralize 0.5 ml of the 2.5% TCA solution (sample solution), 0.2 ml of a solution containing 2 vol 1 M-Tris-HCl (pH 8.3) and 1 vol 1 M-NaOH was added. Thereafter, 5 ml of a solution containing 2% (v/v) pyridine in methanol, 100 mg/l pyridoxamine-di-HCl and 2.0 g/l zinc acetate was added. The reaction of pyridoxamine, 2-oxoglutarate and Zn(II)ion was found to yield a Zn(II) chelate of the aldimine (Matsushima & Martell, 1967). After incubation for 30 min at 37°C the reaction mixture was cooled in ice-water for 3-5 min. The fluorescence intensity was measured within 2 h at room temperature using a Baird-Atomic spectrophotofluorometer with excitation at 395 nm and emission at 475 nm. Standards and blanks were carried throughout the entire assay procedure. Blanks contained the complete incubation mixture, except SSA. The standards were prepared in the same way as blanks and 2-OG was added to final concentrations of 20-80 µM. The change in the glutamate concentration used in the GABA-T assay resulted in blanks which varied to a maximum of 20% and this variation was proportional to the glutamate concentration. Therefore, the assay was always performed with standards of all glutamate concentrations used. Incubation mixtures, to which the TCA solution was added immediately after starting the reaction by addition of SSA, were used as additional controls. When 2-OG was incubated under the conditions of the assay in the absence of SSA it was not significantly metabolized, as measured after 1 h (recovery 98.4 $\pm$ 1.3%; n = 13).

SSA-DH assay.

Measurement of the SSA-DH activity was performed as described previously (De Boer & Bruinvels, 1977a), using a tissue concentration of 0.44 mg/ml (wet wt.).

Data analysis

The apparent kinetic parameters of the forward reaction of GABA-T were calculated from Lineweaver-Burk plots.

Since in both the assay of the reverse reaction of GABA-T and in the assay of SSA-DH more than 5% of the initial concentration of SSA was converted, the apparent kinetic constants were calculated using the integrated form of the Henri-Michaelis-Menten equation (Segel, 1975).

To obtain limiting Michaelis constants for the substrates of GABA-T from the apparent constants the equations for a Ping Pong Bi Bi mechanism (Cleland, 1963) were applied, since it is generally accepted that transaminations occur via such a mechanism (Meister, 1955; Scott & Jakoby, 1959; Henson & Cleland, 1964).

The type of inhibition by DPA of GABA-T was determined graphically. The K_i's were calculated from a replot of the ratio of the apparent K_m and V_{max} vs concentration of inhibitor as suggested by Segel (1975) for mixed-type inhibition.

All constants were calculated from the plots using the least squares method. Variance analysis for regression lines was used to test the significance of differences in K_m and V_{max}.

RESULTS

Kinetics of GABA-T

Reaction with GABA as substrate (forward reaction)

The kinetic constants of the GABA-T forward reaction were determined using concentrations of 0.5-5.0 mM-GABA and 0.05-6.0 mM-2-OG in the absence of NAD. Lineweaver-Burk plots are given in Fig. 1.

Since substrate inhibition was observed with 2-OG (Fig.1A), data obtained with concentrations of 2-OG exceeding 0.5 mM were omitted for calculation of the kinetic constants. The dependency of the apparent kinetic constants for 2-OG on the concentration of GABA, and vice versa, is shown in the plots of Figs. 1A and D. The data were fitted to equation (1)

$$\frac{1}{v} = \frac{1}{V} \times \left[\frac{K_{2\text{-OG}}}{(2\text{-OG})} + \frac{K_{GABA}}{(GABA)} \left(1 + \frac{(2\text{-OG})}{Ki_{2\text{-OG}}} \right) + 1 \right] \qquad (1)$$

where v is the measured velocity, depending on the substrate concentrations, V is the maximum velocity under saturated conditions for both substrates. (2-OG) and (GABA) are the concentrations of 2-OG and GABA, respectively, $K_{2\text{-OG}}$ and K_{GABA} are the Michaelis constants for both substrates under saturated conditions and $Ki_{2\text{-OG}}$ is the inhibition constant for 2-OG. This relationship was derived from the rate equation, which is operative in a Ping Pong Bi Bi mechanism (Cleland, 1963), when substrate inhibition occurs with a single substrate (Garces & Cleland, 1969). To obtain the Michaelis constants for saturated conditions, replots were calculated in which the reciprocal apparent constants, $Km_{2\text{-OG}}$, Km_{GABA} and V_{max}, were used as functions of the reciprocal substrate concentrations (Figs. 1B, C, E and F). Equations describing the curves in these figures, derived from equation (1), are indicated in the legend of Table 1. The values of the limiting Michaelis constants obtained from these replots are summarized in Table 1.

Table 1.

Kinetic constants of the GABA-T forward reaction, calculated for saturated conditions

Fig.1	Replot	$K_{2\text{-OG}}$(mM)	K_{GABA}(mM)	V(µmol/g/h)
B	$1/V_{max}$ vs 1/GABA		2.02	35.18
C	$1/Km_{2\text{-OG}}$ vs 1/GABA	0.29	1.11	
E	$1/V_{max}$ vs 1/2-OG	0.33		35.14
F	$1/Km_{GABA}$ vs 1/2-OG	0.12	1.44	
Mean values		0.25	1.5	35.2

Replotting the reciprocal apparent kinetic constants for the GABA-T forward reaction, obtained from Fig. 1A and D, vs the reciprocal concentrations of the fixed substrate allows the calculation of the limiting Michaelis constants. The following equations were applied:

(B) $$\frac{1}{V_{max}} = \frac{V}{K_{GABA}} \times \frac{1}{(GABA)} + \frac{1}{K_{(GABA)}}$$

(E) $$\frac{1}{V_{max}} = \frac{V}{K_{2\text{-OG}}} \times \frac{1}{(2\text{-OG})} + \frac{1}{K_{2\text{-OG}}}$$

(C) $$\frac{1}{Km_{2\text{-OG}}} = \frac{K_{2\text{-OG}}}{K_{GABA}} \times \frac{1}{(GABA)} + \frac{1}{K_{GABA}}$$

(F) $$\frac{1}{Km_{GABA}} = \frac{V}{K_{2\text{-OG}}} \times \frac{1}{(2\text{-OG})} \times \frac{1}{K_{2\text{-OG}}}$$

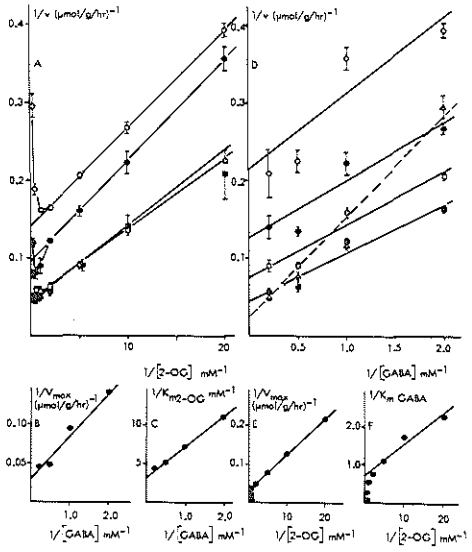

FIG. 1. The kinetic constants of the GABA-T forward reaction. Incubations were performed in 50 mM-Tris-HCl (pH 8.5)-20 mM-2-mercaptoethanol and in the presence of Triton X-100 at a temperature of 22°C. A and D are Lineweaver-Burk plots, of the mean of three experiments, whereas B, C, E and F are replots of the reciprocal apparent kinetic constants obtained from A and D to calculate the limiting Michaelis constants. The reciprocal fixed substrate concentration is given on the horizontal axis. The equations which were applied to calculate the constants are given in the legend to Table 1. The resulting values are summarized in Table 1. A. Reciprocal plot of the velocity vs the concentration of 2-OG with GABA as a fixed substrate. GABA concentrations: o——o, 0.5 mM; ●——●, 1.0 mM; □——□, 2.0 mM; ■——■, 5.0 mM. B. Replot of reciprocal apparent maximum velocity vs the reciprocal GABA concentration. C. Replot of the reciprocal apparent K_m for 2-OG vs the reciprocal GABA concentration. D. Reciprocal plot of the velocity vs the concentration of GABA with 2-OG as fixed substrate. 2-OG concentrations: o——o, 0.05 mM; ●——●, 0.1 mM; □——□, 0.2 mM; ■——■, 0.5 mM; Δ——Δ, 6.0 mM. The broken line for 6.0 mM-2-OG indicates that substrate inhibition occurred. For reasons of simplicity the curves obtained with 2-OG concentrations of 1.0 mM and 2.0 mM are not shown in the figure. E. Replot of the reciprocal apparent maximum velocity vs the reciprocal 2-OG concentration. F. Replot of the reciprocal apparent K_m for GABA vs the reciprocal 2-OG concentration.

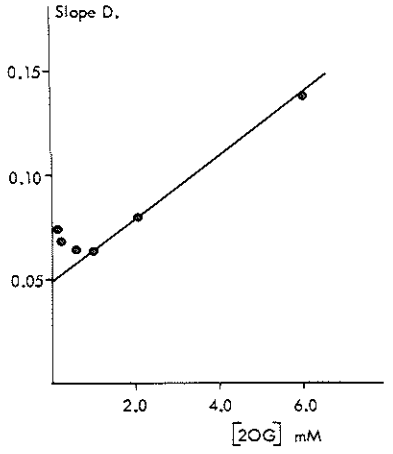

FIG. 2. Replot to determine the K_i for 2-OG as substrate inhibitor. Slopes of the lines given in Fig. 1D were plotted vs the concentration of 2-OG. The following equation was applied to calculate Ki_{2-OG}:

$$\text{Slope D} = \frac{Km_{GABA}}{V_{max} Ki_{2-OG}} (2\text{-OG}) + \frac{Km_{GABA}}{V_{max}}$$

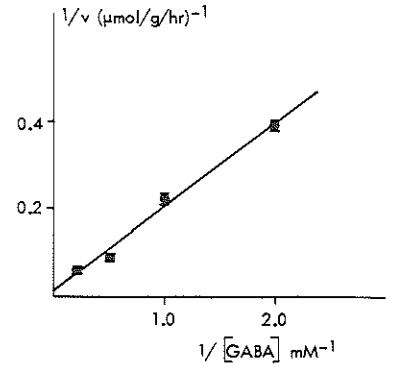

FIG. 3. Reciprocal plot of the velocity vs the concentration of GABA where the (2-OG)/(GABA) ratio is maintained constant (ratio is 0.1). The incubation conditions were the same as in Fig. 1.

A K_i value for 2-OG of 3.3 mM was obtained from a replot of the slopes of the double reciprocal curves (Fig. 1D) vs the concentration of 2-OG according to Segel (1975)(Fig. 2).

For a further verification of the appearance of a Ping Pong Bi Bi mechanism, a reciprocal plot was calculated of the velocity vs the concentration of GABA, in which the concentrations of 2-OG and GABA are varied together with a constant ratio of 0.1 (Fig. 3). A linear relationship was clearly shown. To study the influence of the presence of an active SSA-DH on the activity of GABA-T, the activity of this latter enzyme was measured both in the presence (1.1 mM) and absence of NAD, using 0.5 mM-2-OG (Fig. 4). The rate of conversion of GABA seemed to be higher in the presence than in the absence of NAD (34.2 and 21.9 µmol/g/h, respectively), while the K_m value for GABA was also affected (0.6 and 1.4 mM, respectively). Thus, inhibition of SSA-DH appeared to result in an apparent mixed-type inhibition of GABA-T.

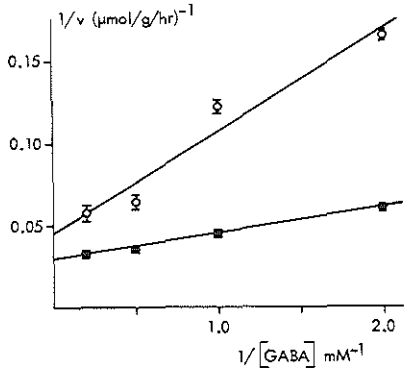

FIG. 4. The effect of inhibition of SSA-DH on the GABA-T forward reaction. Reciprocal plot of the velocity vs the concentration of GABA in the presence of 0.5 mM-2-OG. o——o, in the absence of NAD; ●——●, in the presence of 1.1 mM-NAD to obtain irreversible conditions.

Effect of DPA

Using a concentration of 0.5 mM-2-OG, DPA exerted a weak inhibitory action on GABA-T activity. DPA concentrations used in these experiments were 10, 20 and 40 mM. As can be seen in Fig. 5 this effect was observed with respect to a control containing 40 mM-sodiumisethionate, a concentration equivalent to 40 mM-Na-DPA.

The inhibition was found to be competitive with respect to GABA, with a K_i for DPA of 23.2 mM. Addition of sodiumisethionate to the incubation mixture resulted in an apparent activation of GABA-T through lowering of the K_m of

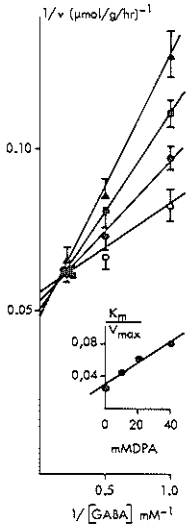

FIG. 5. The effect of DPA on the GABA-T forward reaction. Reciprocal plot of the velocity vs the concentration of GABA in the presence of 0.5 mM-2-OG. o——o, Control in the presence of 40 mM-sodium-isethionate; ●——●, 10 mM-DPA; ■——■, 20 mM-DPA; ▲——▲, 40 mM-DPA. A replot of the ratios of the apparent K_m and V_{max} values vs the DPA concentration is inserted, showing the apparent K_i of DPA.

GABA (0.5 mM in the presence and 1.5 mM in the absence of sodium-isethionate), whereas the V_{max} only slightly differed (17.8 and 21.9 µmol/g/h, respectively). With respect to the control without salt addition (not shown in Fig. 5) there appeared to be hardly any inhibition, as indicated by the differences in K_m and V_{max} in the presence (40 mM) or absence of DPA: Km_{GABA} was 1.7 mM and 1.4 mM, respectively, and the V_{max} was 20.4 and 21.9 µmol/g/h, respectively.

Reaction with SSA as substrate (reverse reaction)

The kinetics of GABA-T using SSA and glutamate as substrates were studied using concentrations of 50-600 µM-SSA and 0.5-5.0 mM-Glu. A typical experiment is shown in Fig. 6. The replots of reciprocal K_m or V_{max} vs reciprocal substrate concentration shown are the means of four experiments (Figs. 6B, C, E and F). As with the forward reaction, two characteristics of the Ping Pong Bi Bi mechanism were also observed in these replots. Thus, when the concentration of one substrate, SSA or Glu, was limiting to zero, the K_m for the other substrate, Glu or SSA, respectively, also decreased to zero. On the other hand, by increasing the concentration of one substrate a limiting value for the K_m of the other substrate could be obtained, indicating saturated conditions. In Table 2 the extrapolated values for the K of both substrates and the V are shown. Concentrations of glutamate exceeding 5.0 mM were found to be inhibitory as measured in the initial two experiments. These high concentrations were, therefore, not used in the two subsequent experiments. Additional evidence that the reaction catalysed by GABA-T obeyed the Ping Pong Bi Bi mechanism was obtained by measuring the inhibition of the reverse reaction by GABA (Fig. 7).

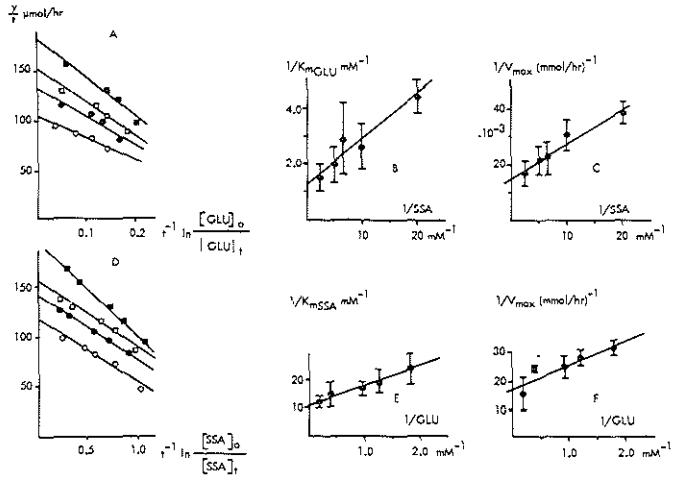

FIG. 6. The kinetic constants of the GABA-T reverse reaction.
The incubations were performed in 50 mM-Tris-HCl (pH 8.5) at 22°C in the presence of 20 mM-2-mercaptoethanol after preincubation of the tissue in 0.5% Triton X-100. A and D are integrated Henri-Michaelis-Menten curves, calculated for a typical experiment. Y is equal to the amount of product formed during the incubation time t. $(SSA)_0$ and $(GLU)_0$ are the initial substrate concentrations, whereas $(SSA)_t$ and $(GLU)_t$ are the substrate concentrations at the end of the incubation period. The slope of the lines obtained from these curves equals $-K_m$, while the intercept with the vertical axis equals the apparent V_{max}. B, C, E and F are replots of the reciprocal apparent kinetic constants, which are the mean of four experiments, obtained from plots similar to A and D. The reciprocal fixed substrate concentration is given on the horizontal axis. The equations which were applied to calculate the limiting Michaelis constants from these plots are given in the legend to Table 2. The resulting values are summarized in Table 2. A. Curves with Glu as varied substrate and SSA as fixed substrate. SSA concentrations: o——o, 0.10 mM; ●——●, 0.15 mM; □——□, 0.20 mM; ■——■, 0.40 mM. B. Replot of the reciprocal apparent K_m for Glu vs the reciprocal SSA concentration. C. Replot of the reciprocal apparent maximum velocity vs the reciprocal SSA concentration. D. Curves with SSA as varied substrate and Glu as fixed substrate. Glu concentrations: o——o, 0.5 mM; ●——●, 0.75 mM; □——□, 1.0 mM; ■——■, 2.5 mM. E. Replot of the reciprocal apparent K_m for SSA vs the reciprocal Glu concentration. F. Replot of the reciprocal apparent maximum velocity vs the reciprocal Glu concentration.

Table 2.

Kinetic constants of the GABA-T reverse reaction calculated for saturated conditions

Fig. 6	Replot	K_{SSA}(μM)	K_{Glu}(μM)	V(μmol/g/h)
B	$1/Km_{Glu}$ vs 1/SSA	123.3	781	
C	$1/V_{max}$ vs 1/SSA	83.8		67.5
E	$1/Km_{SSA}$ vs 1/Glu	55.2	604	
F	$1/V_{max}$ vs 1/Glu		480	58.5
Mean values		87	620	63

Apparent kinetic constants were calculated from four experiments as indicated for a typical experiment in Fig. 6A and D. The mean value obtained for each concentration of the fixed substrate used was reciprocally replotted vs the reciprocal of the concentration of the fixed substrate. An endogenous concentration of 44.0 μM-glutamate in the incubation medium was assumed, based on a total brain concentration of 10 μmol/g tissue (wet wt.) (Van den Berg et al., 1975). The following equations were applied:

(B) $$\frac{1}{Km_{Glu}} = \frac{K_{Glu}}{K_{SSA}} \times \frac{1}{(SSA)} + \frac{1}{K_{SSA}}$$

(C) $$\frac{1}{V_{max}} = \frac{V}{K_{SSA}} \times \frac{1}{(SSA)} + \frac{1}{K_{SSA}}$$

(E) $$\frac{1}{Km_{SSA}} = \frac{K_{SSA}}{K_{Glu}} \times \frac{1}{(Glu)} + \frac{1}{K_{Glu}}$$

(F) $$\frac{1}{V_{max}} = \frac{V}{V_{max}} \times \frac{1}{(Glu)} + \frac{1}{K_{Glu}}$$

The inhibition was shown to be competitive with respect to glutamate (Fig. 7A) while that with respect to SSA was non-competitive (Fig. 7B). The apparent K_i for GABA was 0.60 mM.

Effect of DPA

The effect of DPA on the reverse reaction was measured using concentrations of 1, 5 and 10 mM (Fig. 8). DPA competitively inhibited the conversion of glutamate by GABA-T (Fig. 8A; K_i = 15.2 mM), while the conversion of SSA was inhibited in a non-competitive manner (Fig. 8B; K_i = 22.9 mM).

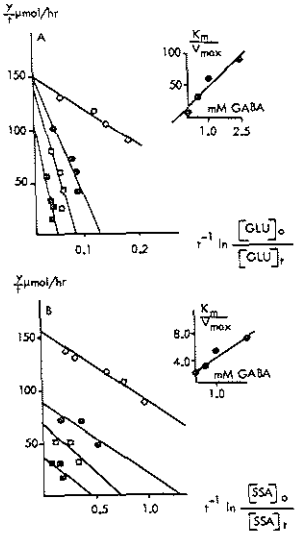

FIG. 7. Effect of GABA on the GABA-T reverse reaction.
Incubations were performed in 50 mM-Tris-HCl (pH 8.5) at 22°C in the presence of 20 mM-2-mercaptoethanol after preincubation of the tissue in 0.5% Triton X-100. Integrated Henri-Michaelis-Menten curves are calculated for a typical experiment. (See also Fig. 6). A. Inhibition of the GABA-T reverse reaction by GABA with respect to Glu. SSA concentration is 0.2 mM. GABA concentrations: o——o, no addition; ●——●, 0.5 mM; □——□, 1.0 mM; ■——■, 2.5 mM. A replot of the ratios of the apparent K_m and V_{max} values vs the GABA concentration is inserted, showing the apparent K_i of GABA. B. Inhibition of the GABA-T reverse reaction by GABA with respect to SSA. Glu-concentration is 1.0 mM. GABA concentrations: o——o, no addition; ●——●, 0.5 mM; □——□, 1.0 mM; ■——■, 2.5 mM. A plot similar to that in A is inserted, showing the apparent K_i of GABA.

Kinetics of SSA-DH

The reaction was carried out using SSA-concentrations of 25-400 µM. Integrated Henri-Michaelis-Menten plots of the data are shown in Fig. 9. Concentrations of SSA higher than 150 µM seemed to be inhibitory and were omitted for the calculation of the K_m and V_{max}. The K_m of SSA for SSA-DH was found to be 9.1 µM, whereas the V_{max} was 27.5 µmol/g/h.

Effect of DPA

Inhibition by DPA was measured using concentrations of 1, 5 and 10 mM of the sodium salt of DPA. A control including 10 mM-sodium-isethionate (equimolar to 10 mM-Na-DPA) was used to reveal possible effects of sodium ions. The inhibition of SSA-DH by DPA appeared to be competitive with respect

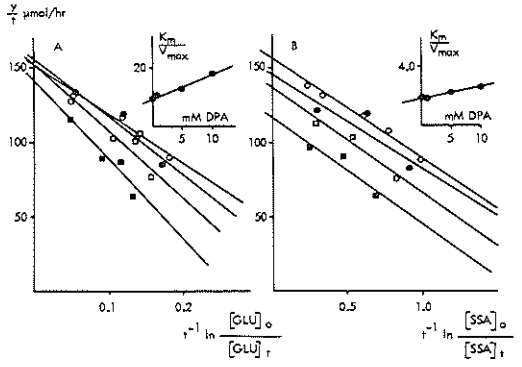

FIG. 8. Integrated Henri-Michaelis-Menten curves, calculated for a representative experiment, to determine the effect of DPA on the reverse reaction of GABA-T. The incubation conditions were the same as in Fig. 6. Inhibition with DPA with respect to Glu, SSA concentration is 0.2 mM. DPA concentrations: o——o, no addition; ●——●, 1 mM; □——□, 5 mM: ▩——▩, 10 mM. A replot to determine the K_i of DPA with respect to glutamate is inserted, in which the ratio of the apparent K_m and V_{max} as a function of the DPA concentration is given. B. Inhibition of DPA with respect to SSA. Glu concentration is 1.0 mM. The symbols are identical to those in A. A similar replot to determine the K_i of DPA with respect to SSA is inserted.

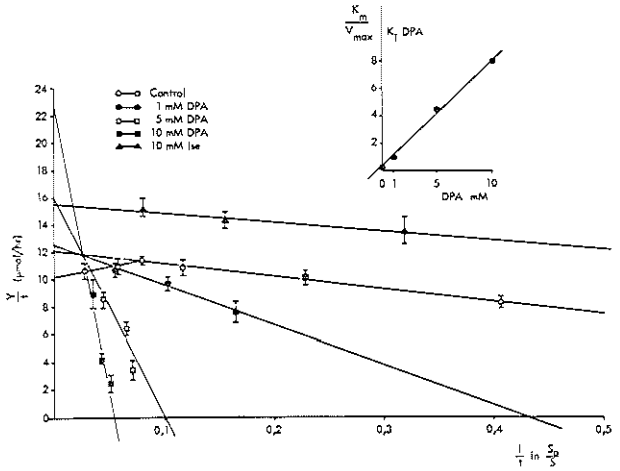

FIG. 9. Integrated Henri-Michaelis-Menten curves to determine the kinetic constants of SSA-DH and the effect of DPA. The incubation was carried out with 0.44 mg/ml tissue (wet wt.) in 50 mM-Tris-HCl (pH 8.5) at 22°C in the presence of 8.2 mM-2-mercaptoethanol. Bars represent S.E.M. (n=3). o——o, No addition; ●——●, 1 mM-DPA; □——□, 5 mM-DPA; ▩——▩, 10 mM-DPA; ▲——▲, control with 10 mM-Na-isethionate. A replot to determine the K_i of DPA with respect to SSA is inserted, in which the ratio of the apparent K_m and V_{max} as a function of the DPA concentration is given.

to SSA (Fig. 9) K_i = 0.5 mM. However, by increasing DPA concentrations the V_{max} was also increased (V_{max} = 51.5 µmol/g/h). Such an enhancement of the activity was also observed in the control containing sodium-isethionate (V_{max} = 35.6 µmol/g/h). The apparent difference between this latter enhancement and the DPA-induced increase of the activity was not statistically significant.

DISCUSSION

Administration of DPA (400 mg/kg, i.p.) increases GABA concentrations in rat brain up to 140% (Godin et al., 1969; Horton et al., 1977; Schwarcz et al., 1977) while a similar increase was observed after oral administration of the same dose (Kukino & Deguchi, 1877). The present results indicate that inhibition of SSA-DH by DPA is responsible for this increase in GABA.

Several authors have simultaneously determined the K's of GABA and 2-OG for GABA-T in several species including rabbit, mouse and bovine (Waksman & Roberts, 1965; Schousboe et al., 1973; Simler et al., 1973; John & Fowler, 1976; Tunnicliff & Ngo, 1977; Kobayashi et al., 1977; Maitre et al., 1978).

Three reports concerning rat brain GABA-T are known (Pitts et al., 1965; Sytinski & Vasilijev, 1970; Maitre et al., 1975), in which almost identical affinities of the substrates 2-OG and GABA for the enzyme GABA-T were reported, while the present results revealed a K for GABA which was 6 times higher than that for 2-OG. This discrepancy is probably due to different incubation conditions.

The data indicated that at high 2-OG concentration a dead-end 2-OG-enzyme complex is formed (Fig. 1A and D). Although several authors have observed substrate inhibition of the forward reaction by 2-OG in several species (Scott & Jakoby, 1959; Hall & Kravitz, 1967; Sytinski & Vasilijev, 1970; Tunnicliff & Ngo, 1977; Maitre et al., 1978), only Maitre et al. (1975) reported results comparable with the present data. Recalculation of the data obtained by Maitre et al. (1975) according to Segel (1975) resulted in a K_i for 2-OG of approx 12 mM, while the authors themselves reported a value of 0.18 mM. This recalculated K_i is of the same order of magnitude as that calculated from the present data (3.3 mM).

The observation that 2-OG could be quantitatively recovered after 1 h of incubation using assay conditions for the reverse reaction indicated the absence of any significant 2-OG converting aminotransferase activity (e.g. aspartate aminotransferase) as suggested by Maitre et al. (1976). While

GABA and 2-OG both exhibited higher affinity for GABA-T during the forward reaction, as discussed above, the affinities of SSA and Glu in the reverse reaction were also higher than those reported by others (Harvey et al., 1975; Maitre et al., 1975).

The K_i of GABA (0.6 mM) must be viewed as an apparent value measured at a fixed concentration of Glu (1.0 mM) or SSA (0.2 mM). Nevertheless, it might be a good estimate of the real value since the concentrations of both substrates used exceeded their K values for GABA-T.

It seems remarkable that the K_i of GABA for the reverse reaction, which represents the extent of binding of GABA to the aldimine form of GABA-T without subsequent conversion in competition with respect to Glu, is lower than the K of this substance for the forward reaction, which represents the extent of GABA converted into SSA by the enzyme. This suggests that in the overall conversion of GABA into SSA and the concomitant conversion of the aldimine form of the enzyme into the ketimine form, the binding of GABA to the enzyme does not represent the rate-limiting step.

This interpretation is consistent with the results of John & Fowler (1976), who reported that the conversion of the GABA-enzyme complex from the aldimine into the ketimine form is relatively slow as compared with identical steps in other transaminase catalysed reactions.

The enzyme kinetics of GABA-T presented are consistent with a Ping Pong Bi Bi mechanism, which is generally accepted to be the underlying mechanism for transamination reactions. According to Segel (1975) the family of parallel lines (Fig. 1A), as well as the linear reciprocal plot when the concentrations of 2-OG and GABA are varied together with a constant ratio (Fig. 3) are sufficient for the identification of a Ping Pong Bi Bi mechanism.

An ordered Bi Bi mechanism, which may yield similar results under certain conditions, can be rejected, since the inhibition pattern of GABA in the reverse reaction does not fit in with this latter system. Four equations, relating the equilibrium constant and the various kinetic constants (Haldane relationships) are operative in the Ping Pong Bi Bi mechanism. The following equation provides an indication of the occurrence of the reverse reaction in this system.

$$K_{eq} = \left(\frac{V_f}{V_r}\right)^2 \times \frac{K_{SSA}K_{Glu}}{K_{GABA}K_{2\text{-}OG}} = 0.04 \qquad (2)$$

V_f and V_r are the velocities of the forward reaction and reverse reaction respectively. Two values for the K_{eq} of GABA-T from different species have

been reported: for mouse brain GABA-T, K_{eq} = 0.29 (Duffy et al., 1972), and for *Pseudomonas fluorescens* GABA-T, K_{eq} = 0.10 (Scott & Jakoby, 1959). The latter two K_{eq}'s have been determined by measuring the concentrations of the substrates when the reaction was allowed to come into equilibrium, applying the following equation:

$$K_{eq} = \frac{(GABA)(2\text{-}OG)}{(SSA)(GLU)} \qquad (3)$$

Scott & Jakoby (1959) have reported the effect of different initial substrate concentrations on the resulting K_{eq}. Since the K_{eq}, calculated with equation (2) is independent of the substrate concentrations, this value seems to be more reliable. Besides a species difference, it might be possible that the discrepancy in these K_{eq} values and the present value is due to a different way of calculating the K_{eq}.

The present observation that omission of NAD from the incubation mixture resulted in an impaired GABA-T activity during the assay of the forward reaction (Fig. 4) is in agreement with the findings of Hall & Kravitz (1967) using lobster GABA-T, and can be taken as indirect evidence for an equilibrium biased towards GABA.

Although the value of the K_{eq} strongly suggests that the equilibrium is biased towards GABA, it seems doubtful whether this mechanism will contribute to the *in vivo* synthesis of GABA, since the substrate for the reverse reaction, SSA, is continuously removed by a subsequent almost irreversible reaction via SSA-DH (Pitts & Quick, 1965). Although the present value of the V_{max} of SSA-DH is not very high, it is not directly comparable with the value of the V of GABA-T, since somewhat different conditions were used.

Recent studies have indicated that monovalent and bivalent cations can affect the activity of SSA-DH (De Boer & Bruinvels, 1977b). Therefore, it is conceivable that, *in vivo*, these ions exert a regulating role in the metabolism of SSA and will therefore indirectly affect the formation of GABA via the reverse reaction of GABA-T.

Effect of DPA

The preferential action of DPA on SSA-DH, as shown by its higher affinity for this enzyme compared to that for GABA-T, is in agreement with the findings of Harvey et al. (1975) and of Anlezark et al. (1976). Only a slight inhibitory effect of DPA on the GABA-T forward reaction was found using conditions which excluded interference of DPA with SSA-DH. The K_i of DPA (23.2 mM) for this

reaction is in the same order of magnitude as those found by Maitre et al. (1974) for mouse brain GABA-T (approx 18 mM) and Fowler et al. (1975) for rabbit brain GABA-T (42 mM) using an assay in which SSA is measured directly. Recently, Kukino & Deguchi (1977) reported only a 10% inhibition of rat brain GABA-T using 60.2 mM-DPA. The lower values for the K_i of DPA reported by Maitre et al. (1976) for rat brain (9.5 mM) and Simler et al (1973) for mouse brain (1.4 mM) may be due to simultaneous inhibition of SSA-DH, since these authors have used a coupled enzyme assay.

In preliminary experiments, using the coupled assay of De Boer & Bruinvels, (1977a). omission of NAD from the reaction mixture, which inhibited SSA-DH completely, and addition of 10 mM-DPA in the presence of NAD gave the same results (unpublished).

As shown in Fig. 4, inhibition of SSA-DH by omission of NAD from the incubation mixture also resulted in an inhibition of GABA-T, indicating that this inhibition is not the result of a direct effect on GABA-T.

The K_i of DPA for the reverse reaction catalysed by GABA-T in the present study (15.2 mM with respect to glutamate and 22.9 mM with respect to SSA), was of the same order of magnitude as that reported by Godin et al. (1969). The difference between the K_i values for DPA with respect to Glu and SSA, on the one hand, and the different type of inhibition on the other hand, indicate that the affinity of DPA for the aldimine form is higher than that for the ketimine form of the enzyme. This has also been reported by Fowler et al. (1975) using rabbit brain GABA-T.

This higher affinity of DPA for the aldimine form of the enzyme seems surprising since the carboxyl group of the amino acids, which is thought to be equivalent to the carboxyl group of DPA, appears to be of minor importance in the specific binding to the aldimine form (Snell & Di Mari, 1970).

The present experiments revealed a K_i of DPA for SSA-DH of 0.5 mM, which is more or less consistent with K_i's reported by Harvey et al. (1975) and Anlezark et al. (1976), namely 1.5 mM and a value below 1 mM, respectively. However, a higher K_i (4 mM) was reported by Maitre et al. (1976). Nevertheless, it can be concluded that DPA has a much higher affinity for SSA-DH than for GABA-T.

The increase in the apparent V_{max} for SSA-DH observed after addition of DPA must be ascribed to the presence of sodium ions in the incubation medium, since in the control experiment in which an equimolar concentration of sodium-isethionate was used instead of the Na-DPA salt, a comparable increase in the V_{max} was shown.

As discussed, the K_i values for GABA-T (reverse reaction) and SSA-DH differ considerably, being 0.5 mM for SSA-DH and 22.9 mM for GABA-T. Consequently, it may be expected that DPA, administered *in vivo*, might primarily cause a strong inhibition of SSA-DH, since the concentration of DPA in brain will reach only low values, of the order of magnitude of the K_i of DPA for SSA-DH, whereas much higher concentrations of DPA are required for significant inhibition of GABA-T. It has been reported that plasma levels of DPA in man are 0.07-1.0 mM (Sawaya et al., 1975). In view of the reports that brain levels of DPA are approx 30% of the plasma level in mice (Löscher & Frey, 1977; Schobben & Van der Kleyn, 1974) and monkeys (Schobben et al., 1977), these data favour a preferential action of DPA on SSA-DH in vivo.

The affinity of SSA-DH, which was shown to differ markedly from the affinity of SSA for GABA-T, would be expected to decrease in the presence of DPA to the same value or even lower than that obtained for GABA-T. Therefore, addition of DPA will cause accumulation of SSA, thus initiating the conversion of SSA into GABA via the reverse reaction of GABA-T.

In contrast to the present results, Maitre et al. (1976) reported that addition of p-hydroxybenzaldehyde, a potent SSA-DH inhibitor, *in vitro*, does not change the conversion of GABA into SSA by GABA-T. However, from their experiments it can be calculated that less than 10 µM-SSA was formed during the incubation, a concentration which is far below the K of SSA for GABA-T (87 µM) to initiate the reverse reaction of GABA-T.

An additional mechanism by which DPA might contribute to an increase in GABA concentration in brain is inhibition of the GABA-T forward reaction by the DPA induced accumulation of SSA. The present data show that omission of NAD from the incubation mixture, resulting in an inactive SSA-DH, greatly reduced (30%) the measured activity of GABA-T. SSA accumulated during the incubation period of 30 min, reaching a final concentration of 30-40 µM. This concentration would be sufficient to cause product inhibition but might be too low to explain the increase in GABA via conversion of SSA. From a Haldane equation, which is an equivalent of equation (2), the K_i of SSA for GABA-T could be calculated as follows:

$$Ki_{SSA} = K_{eq} \frac{V_r}{V_f} \times \frac{Ki_{GABA}\, K_{2\text{-}Og}}{K_{Glu}} = 19\ \mu M$$

This value is a rough estimate of the real value but it provides an indication of the order of magnitude. The value is quite low if compared with known data. Maitre et al. (1975) have found a competitive inhibition of GABA-T by SSA with respect to GABA with a K_i of 4 mM. However, this type of inhibition does not fit with the Ping Pong Bi Bi mechanism, since in this system non-competitive inhibition has to be expected. Dye & Taberner (1975) were not able to find any inhibitory effect of SSA using concentrations of up to 20 mM. These authors, however, did not use Triton X-100 in their incubation mixture to obtain maximal activity of the enzyme.

The present results indicate that two mechanisms can be held responsible for the DPA induced increase in GABA. The primary effect is inhibition of SSA-DH and will result in accumulation of SSA, which will inhibit the forward reaction of GABA-T preventing the degradation of GABA. A further increase of SSA will initiate the conversion of SSA into GABA by the reverse reaction of GABA-T. Thus, DPA will act indirectly as an inhibitor of the GABA-T forward reaction. A schematic presentation summarizing the mechanism involved in the action of GABA and SSA is shown in Fig. 10.

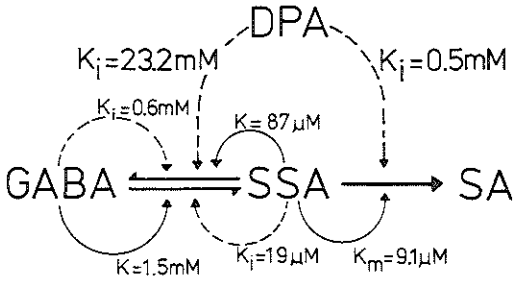

FIG. 10. Limiting Michaelis constants and inhibition constants of GABA, SSA and DPA for GABA-T and SSA-DH. The unbroken lines represent the Michaelis constants, indicating the affinity of the substrate for the binding to the enzyme resulting in conversion. The broken lines represent the inhibition constants, indicating the affinity of the substrates (or products) for binding to the enzyme not resulting in conversion. The inhibition constant for DPA is also represented, as a broken line.

Although many authors ascribed the anticonvulsant action of DPA to an enhanced GABA level (Godin et al., 1969; Anlezark et al., 1976; Löscher & Frey, 1977), which has been shown to be correlated (Simler et al., 1973; Wood et al., 1977; Lust et al., 1978), it was not until now clearly understood

how DPA exerts its action, because of its weak inhibitory effect on GABA-T (Kukino & Deguchi, 1977; Fowler et al., 1975). The action of DPA on SSA-DH and the effects of the resulting accumulated SSA might explain the increase in GABA levels in a more satisfactory way. It is interesting to note that inhibitors of GABA-T like AOAA exert their action via trapping of pyridoxal phosphate and therefore the action of GAD, which also needs this cofactor, will be impaired too. Inhibition of SSA-DH might, therefore, be a better choice to increase GABA levels *in vivo*.

REFERENCES

Anlezark,G., Horton,R.W., Meldrum,B.S., Sawaya,M.C.B.(1976) Anticonvulsant action of ethanolamine-O-sulphate and di-n-propylacetate and the metabolism of γ-aminobutyric acid (GABA) in mice with audiogenic seizures. Biochem. Pharmac. 25, 413-417

Baxter, C.F., Roberts, E.(1961) Elevation of γ-aminobutyric acid in brain: selective inhibition of γ-aminobutyric-α-ketoglutaric acid transaminase. J. Biol. Chem. 236, 3287-3294

Cleland, W.W. (1963) The kinetics of enzyme-catalyzed reactions with two or more substrates or products 1. Nomenclature and rate equations. Biochim. Biophys. Acta 67, 104-137

De Boer, Th., Bruinvels,J. (1977a) Assay and properties of 4-aminobutyric-2-oxoglutaric acid transaminase and succinic semialdehyde dehydrogenase in rat brain tissue J. Neurochem. 28, 471-478

De Boer, Th., Bruinvels, J. (1977b) Succinic semialdehyde dehydrogenase: activation by monovalent and bivalent cations. Proc. Int. Soc. Neurochem. 6, 524

Duffy, T.E., Nelson, S.R., Lowry, O.H. (1972) Cerebral carbohydrate metabolsim during acute hypoxia and recovery. J. Neurochem. 19, 959-977

Dye, D.J., Taberner, P.V. (1975) The effect of some newer anaesthetics on the *in vitro* activity of glutamate decarboxylase and GABA transaminase in crude brain extracts and on the levels of amino acids *in vivo*. J. Neurochem. 24, 997-1001

Fowler, L.J., John, R.A. (1972) Active-site-directed irreversible inhibition of rat brain 4-aminobutyrate amino-transferase by ethanolamine-O-sulphate *in vitro* and *in vivo*. Biochem. J. 130, 569-573

Fowler, L.J., Beckford, J., John, R.A. (1975) An analysis of the inhibition of rabbit brain γ-aminobutyrate aminotransferase by sodium n-dipropyl-acetate and some other simple carboxylic acids. Biochem. Pharmac. 24, 1267-1270

Garces, E., Cleland, W.W. (1969) Kinetic studies of yeast nucleotide di-phosphate kinase. Biochemistry, Easton 8, 633-640

Godin, Y., Heiner, L., Mark., J., Mandel, P. (1969) Effects of di-n-propyl-acetate, an anticonvulsive compound, on GABA metabolsim. J. Neurochem. 16, 869-873

Hall, Z.W., Kravitz, E.A. (1967) The metabolism of γ-aminobutyric acid (GABA) in the lobster nervous system-1. GABA-glutamate transaminase. J. Neurochem. 14, 45-54

Harvey, P.K.P., Bradford, H.F., Davison, A.N. (1975) The inhibitory effect of sodium n-dipropylacetate on the degradative enzymes of the GABA shunt. FEBS Lett. 52, 251-254

Henson, C.P., Cleland, W.W. (1964) Kinetic studies of glutamic oxaloacetic transaminase isozymes. Biochemistry, Easton 3, 338-345
Horton, R.W., Anlezark, G.M., Sawaya, M.C.B., Meldrum, B.S. (1977) Monoamine and GABA metabolism and the anticonvulsant action of di-n-propylacetate and ethanolamine-O-sulphate. Eur. J. Pharmac. 41, 387-397
John, R.A., Fowler, L.J. (1976) Kinetic and spectral properties of rabbit brain 4-aminobutyrate aminotransferase. Biochem. J. 155, 645-651
Jung, M.J., Lippert, B., Metcalf, B.W., Schechter, P.J., Böhlen, P., Sjoerdsma, A. (1977) The effect of 4-amino-hex-5-ynoic acid (γ-acetylenic GABA, γ-ethynyl GABA) a catalytic inhibitor of GABA metabolism *in vivo*. J. Neurochem. 28, 717-723
Kobayashi, K., Miyazawa, S., Endo, A. (1977) Isolation and inhibitory activity of gabaculine, a new potent inhibitor of γ-aminobutyrate aminotransferase produced by a streptomyces. FEBS Lett. 76, 207-210
Krnjević, K. (1970) Glutamate and γ-aminobutyric acid in brain. Nature, Lond. 228, 119-124
Kukino, K., Deguchi, T. (1977) Effects of sodium dipropylacetate on γ-aminobutyric acid and biogenic amines in rat brain. Chem. Pharm. Bull., Tokyo 25, 2257-2262
Löscher, W., Frey, H.H. (1977) On the mechanism of action of valproic acid. Arzneim. Forsch./Drug Res. 27, 1081-1082
Lust, W.D., Kupferberg, H.J., Yonekawa, W.D., Penry, J.K., Passonneau, J.V., Wheaton, A.B. (1978) Changes in brain metabolites induced by convulsants or electroshock: effects of anticonvulsant agents. Molec. Pharmac. 14, 347-356
Maitre, M., Cieselski, L., Mandel, P. (1974) Effect of 2-methyl-2-ethylcaproic and 2-2-dimethyl valeric acid on audiogenic seizures and brain gamma-aminobutyric acid. Biochem. Pharmac. 23, 2363-2368
Maitre, M., Cieselski, L., Cash, C.,Mandel, P. (1975) Purification and studies on some properties of the 4-aminobutyrate: 2-oxoglutarate transaminase from rat brain. Eur. J. Biochem. 52, 157-169
Maitre, M., Ossola, L., Mandel, P. (1976) *In vitro* studies into the effect of inhibition of rat brain succinic semialdehyde dehydrogenase on GABA-synthesis and degradation. FEBS Lett. 72, 53-57
Maitre, M., Cieselski, L., Cash, C., Mandel, P. (1978) Comparison of the structural characteristics of the 4-aminobutyrate: 2-oxoglutarate transaminases from rat and human brain, and of their affinities for certain inhibitors. Biochim. Biophys. Acta 522, 385-399
Matsushima, Y., Martell, A.E. (1967) Pyridoxal analogues. X. Zinc (II)-chelate catalysis of transamination in methanol solution. J. Am. Chem. Soc. 89, 1331-1335
Meister, A. (1955) Transamination, in Advances in Enzymology (Nord F.F. ed) Vol. 16, pp.185-246. Interscience, New York
Pitts, F.N., Quick, C. (1965) Brain succinate semialdehyde dehydrogenase-1, assay and distribution. J. Neurochem. 12, 893-900
Pitts, F.N., Quick, C., Robins, E. (1965) The enzymatic measurement of γ-aminobutyric-α-oxoglutaric transaminase. J. Neurochem. 12, 93-101
Sawaya, M.C.B., Horton, R.W., Meldrum, B.S. (1975) Effects of anticonvulsant drugs on the cerebral enzymes metabolizing GABA. Epilepsia 16, 649-655
Schechter, P.J., Tranier, Y., Jung, M.J., Böhlen, P. (1977) Audiogenic seizure protection by elevated brain GABA concentration in mice: effects of γ-acetylenic-GABA and γ-vinyl-GABA, two irreversible GABA-T inhibitors. Eur. J. Pharmac. 45, 319-328
Schobben, F., Van der Kleyn, E. (1974) Pharmacokinetics of distribution and elimination of sodium di-n-propylacetate in mouse and dog. Pharm. Weekblad 109, 33.42

Schobben, F., Van der Kleyn, E., Vree, T.B., Guelen, P.J.M. (1977) Pharmacokinetics of dipropylacetate (2-n-propyl pentanoate) in man and laboratory animals, in Antiepileptic Drug Monitoring. (Gardner-Thorpe, C., Janz, D., Meinardi, H., Pippenger, C. eds.) pp. 147-169 Pitman, Kent
Schousboe, A., Wu, J.-Y., Roberts, E. (1973) Purification and characterization of the 4-aminobutyrate-2-ketoglutarate transaminase from mouse brain. Biochemistry, Easton 12, 2868-2873
Schwarcz, R., Bennett, J.P., Coyle, J.T. (1977) Inhibitors of GABA metabolism: implications for Huntington's disease. Ann. Neurol. 2, 299-303
Scott, E.M., Jakoby, W.B. (1959) Soluble γ-aminobutyric-glutamic transaminase from Pseudomonas fluorescens. J. Biol. Chem. 234, 932-936
Segel, I.H. (1975) Enzyme Kinetics. Wiley, New York
Simler, S., Cieselski, L., Maitre, M., Randrianarisoa, H., Mandel, P. (1973) Effect of sodium n-dipropylacetate on audiogenic seizures and brain γ-aminobutyric acid level. Biochem. Pharmac. 22, 1701-1708
Snell, E.E., Di Mari, S.J. (1970) Schiff base intermediates in enzyme catalyses, in The Enzymes (Boyer, P.D. ed.), 3rd Ed., Vol. 2, pp. 335-370. Academic Press, New York
Sytinski, I.A., Vasilijev, V.Y. (1970) Some catalytic properties of purified γ-aminobutyrate-α-oxoglutarate transaminase from the rat brain. Enzymologia 39, 1-11
Takeda, M., Kinoshita, T., Tsuji, A. (1976) A fluorophotometric method for the determination of α-oxoacids by using pyridoxamine and Zinc (II)ion. Analyt. Biochem. 72, 184-190
Tapia, R. (1975) Biochemical pharmacology of GABA in CNS, in Handbook of Psychopharmacology (Iversen, L.L., Iversen, S.D., Snyder, S.H., eds.) Vol. 4, pp. 1-58. Plenum Press, New York
Tower, D.B. (1976) GABA and seizures: clinical correlates in man, in GABA in Nervous System Function. (Roberts, E., Chase, T.N., Tower, D.B. eds.) pp. 461-478. Raven Press, New York
Tunnicliff, G., Ngo, T.T. (1977) The mode of action of homocysteine on mouse brain glutamic decarboxylase and γ-aminobutyrate aminotransferase. Can. J. Biochem. 55, 1013-1018
Van den Berg, C.J., Matheson, D.F., Ronda, G., Reynierse, G.L.A., Blokhuis, G.C.D., Kroon, M.C., Clarke, D.D., Garfinkel, D. (1975) A model of glutamate metabolism in brain: a biochemical analysis of a heterogenous structure, in Metabolic Compartmentation and Neurotransmission. NATO Advanced Study Institute Series, Vol. 6 (Berl., S., Clarke, D.D., Schneider, D., eds.) pp. 515-543. Plenum Press, New York
Waksman, A., Roberts, E. (1965) Purification and some properties of mouse brain γ-aminobutyric-α-ketoglutaric acid transaminase. Biochemistry, Easton 4, 2132-2138
Wood, J.D., Durham, J.S., Peesker, S.J. (1977) Effect of di-n-propylacetate and γ-acetylenic GABA on hyperbaric oxygen-induced seizures and GABA metabolism. Neurochem. Res. 2, 707-715

CHAPTER III

EFFECTS OF BRANCHED-CHAIN FATTY ACIDS ON GABA-DEGRADATION AND BEHAVIOUR: FURTHER EVIDENCE FOR A ROLE OF GABA IN QUASI-MORPHINE ABSTINENCE BEHAVIOUR

J.W. van der Laan, A.W.C.M. Jacobs and J. Bruinvels

Published in Pharmacology Biochemistry & Behavior 13 (1980)843-849

ABSTRACT

An increase in GABA-ergic activity has been implicated in the initiation of quasi-morphine abstinence behaviour by di-n-propylacetate (DPA). Two structural analogues of DPA, namely the branched-chain fatty acids 2-methyl 2-ethylcaproic acid and 2,2-dimethylvaleric acid have now been used to study this relationship between behavioural and biochemical effects. A correlation appeared to exist between the K_i of these compounds for succinic semi-aldehyde dehydrogenase, the second enzyme in the degradation of GABA, and the doses exerting a maximum effect on behaviour. On the other hand, concurrent inhibition of GABA-transaminase seemed to suppress the behavioural effects of the fatty acids. This apparent paradox can possibly be explained by supposing a different action of the fatty acids in distinct compartments of the brain, suggesting an important role for increased GABA-ergic activity in the neuronal compartment in the initiation of the quasi-morphine abstinence behaviour.

INTRODUCTION

Elevation of GABA levels by di-n-propylacetate has been shown to occur via inhibition of succinic semialdehyde dehydrogenase (SSA-DH), the second enzyme in the degradation of GABA (Harvey et al., 1975; Van der Laan et al., 1979). The resulting accumulation of succinic semialdehyde (SSA) probably increases GABA concentration by product inhibition of GABA-transaminase (GABA-T). In addition, backward formation of GABA from SSA may occur since the reactions catalysed by GABA-T are strongly biased towards GABA (Van der Laan et al., 1979).

In order to find more evidence for the involvement of this mechanism in DPA-induced "quasi-morphine abstinence"behaviour (De Boer et al., 1977), we have studied the effects of some analogues of DPA both on behaviour and on the enzymes involved in GABA-degradation.

MATERIALS AND METHODS

Behavioural Studies

Animals

Male albino rats (100-200 g), randomly selected from an inbred Wistar strain (TNO, Zeist, The Netherlands), were used for all experiments. The animals were housed four or five to a plastic cage with food and water ad lib. Lights were kept on from 8.30 a.m. till 20.30 p.m. Behavioural observations were performed between 10.00 a.m. and 16.30 p.m. in a room with constant background noise and a constant temperature of 22 $\pm$ 1°C.

Behaviour

Observation of behaviour was carried out essentially as described by De Boer et al. (1977). Briefly, the scoring system of Frederickson and Smits (1973) was used, in which scores of 0 (absent), 2 (mild) or 4 (marked) were given to penile erection, penis licking, ptosis, teeth chattering, swallowing, tremor, hunchback posture and piloerection. Escape digging, body shaking, head shaking and foreleg shaking were counted and assigned scores of 2, 4, 6, 8 or 10 if they occurred 2-5, 6-10, 11-20, 21-40 or more than 40 times, respectively, during the observation period of 15 min. Horizontal activity was measured using a Varimex activity meter.

Drug preparation and administration

Sodium di-n-propylacetate (Sodium Valproas, Albic BV, Maassluis, The Netherlands) was dissolved in deionized water (0.6 mmol/ml). 2-Methyl, 2-ethylcaproic acid (MEC) and 2,2-dimethylvaleric acid (DMV) were a gift from Prof. Dr. A Lespagnol (Lille, France) and were, like caproic acid (Merck, Darmstadt, GFR) dissolved in deionized water, together with an equimolar quantity of sodium hydroxide to obtain a similar solution as for DPA. For intraperitoneal injections, the following concentrations were used: MEC, 0.3 mmol/ml; DMV, 0.4 mmol/ml; and caproate, 1.2 mmol/ml.

Biochemical Studies

Materials

Apart from the drugs mentioned above, the following compounds were used: GABA was obtained from Calbiochem; Triton X-100 and 2-mercaptoethanol were obtained from BDH Chemicals Ltd. SSA was purchased from Sigma Chemical Company. NAD and NADH were obtained from Boehringer Mannheim. (U-^{14}C)GABA (specific radioactivity 224 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. All other chemicals were purchased from Merck, Darmstadt.

Tissue preparation

Randomly selected male Wistar rats (125-175 g) were killed by decapitation and their brains were rapidly removed and weighed. A 10% (w/v) brain homogenate was prepared in an ice-cold solution containing 0.32 M-sucrose and 4.5 mM-2-mercaptoethanol, using a Teflon-glass Potter-Elvehjem homogenizer. One volume of the homogenate was added to 3 vol of ice-cold Triton medium (0.67% w/v Triton X-100, 50 mM-Tris-HCl (pH 8.5) and 4.5 mM-2-mercaptoethanol) and kept in ice water for 1 h before use.

GABA-T assay

For the assay of GABA-T a radiochemical method was used, as previously described (De Boer and Bruinvels, 1977), with the following modifications. NAD was omitted from the incubation mixture to exclude interference with SSA-DH. Tissue concentration was 1.11 mg/ml(wet wt.). Drugs, as mentioned above, were used as their sodium salts and, when necessary, sodium isethionate was added to obtain a standard sodium concentration of 20 mM in all incubation media. After preincubation at 22^{o}C for 30 min, the reaction was started by the addition of homogenate and subsequently the mixture was incubated for a further 60 min at the same bath temperature. All experiments were carried out in duplicate.

SSA-DH assay

Measurement of SSA-DH activity was performed as previously described (De Boer and Bruinvels, 1977) using a tissue concentration of 0.44 mg/ml (wet wt.). In all incubations the sodium concentration used was 10 mM. Al incubations were performed in quadruplicate.

Data analysis

The kinetic parameters of GABA-T were calculated from Lineweaver-Burke plots. Since in the assay of SSA-DH more than 5% of the initial concentration of SSA was converted, the apparent kinetic constants were calculated using the integrated form of the Henri-Michaelis-Menten equation (Segel, 1975).

The type of inhibition of the enzymes by the fatty acids was determined graphically. The K_i's were calculated from a replot of the ratio of the apparent K_m and V_{max} versus the concentration of the inhibitor as suggested by Segel (1975) for mixed type inhibitions. All constants were calculated from the plots using the method of least squares.

RESULTS

Behaviour

As shown in Table 1 and Fig. 1, most behavioural symptoms induced by DPA could also be evoked by the other branched-chain fatty acids, MEC and DMV. In contrast, only a few so-called "checked signs", viz. swallowing, hunchback posture, piloerection and ptosis, could be observed after injection of the short straight-chain fatty acid, caproic acid.

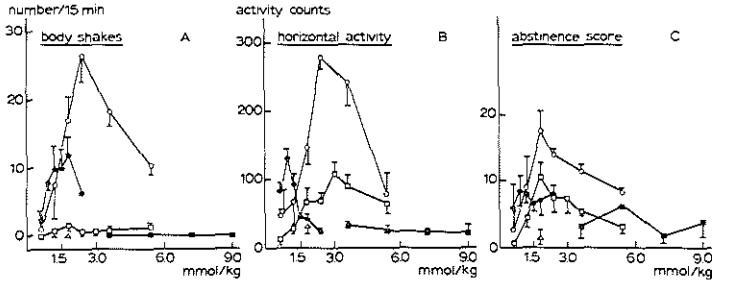

FIG. 1. Dose-effect curves of the behaviour observed after injection of some branched-chain fatty acids or caproate. Rats were treated with increasing doses of DMV, MEC, DPA or caproate after which their behaviour was observed for 15 min. Bars represent SEM. For each compound the same symbol in all figures was used: o —— o, DPA; ● —— ●, MEC; □—— □ , DMV; ■ —— ■ , Caproate; Δ , NaCl.

Table 1. Effect of short-chain and branched-chain fatty acids on behavioural parameters

Compound	Dose mmol/kg	n	Counted Signs		Checked Signs					
			Escape digging	Head shakes	Swallowing	Hunchback posture	Pilo erection	Penis licking	Ptosis	Teeth chattering
DMV	0.6	3	--	1/3	--	1/3	--	--	--	--
	1.2	4	--	--	3/4	1/4	1/4	1/4	2/4	--
	1.8	4	3/4	1/4	3/4	4/4	1/4	3/4	2/4	1/4
	2.4	4	1/4	--	3/4	4/4	2/4	1/4	2/4	2/4
	3.0	4	1/4	2/4	1/4	4/4	1/4	1/4	1/4	1/4
	3.6	4	--	1/4	--	4/4	4/4	--	2/4	--
	5.4	5	--	--	--	2/5	4/5	--	--	--
MEC	0.6	3	--	1/3	--	2/3	1/3	1/3	--	--
	0.9	4	2/4	--	1/4	4/4	2/4	1/4	1/4	--
	1.2	4	--	2/4	--	2/4	4/4	--	--	--
	1.5	4	--	--	--	--	3/4	--	1/4	--
	1.8	4	--	1/4	--	1/4	1/4	--	1/4	--
	2.4	3	--	1/3	--	1/3	2/3	--	1/3	--
DPA	0.6	3	1/3	--	1/3	--	--	1/3	--	1/3
	1.2	4	1/4	2/4	1/4	2/4	3/4	2/4	2/4	--
	1.8	4	3/4	3/4	2/4	4/4	4/4	1/4	2/4	
	2.4	4	2/4	1/4	2/4	4/4	4/4	--	--	--
	3.6	4	--	2/4	--	4/4	3/4	1/4	--	--
	5.4	4	--	1/4	--	4/4	3/4	--	--	--
Caproate	3.6	3	--	--	--	2/3	2/3	--	1/3	--
	5.4	3	--	--	1/3	3/3	3/3	--	2/3	--
	7.2	3	--	--	1/3	2/3	--	--	--	--
	9.0	3	--	--	1/3	2/3	1/3	--	2/3	--
NaCl	1.8	4	1/4	1/4	1/4	--	--	--	--	--

Survey of the abstinence behaviour induced by MEC, DMV, DPA and caproate in rats treated with varying doses and observed for 15 min. For all symptoms the incidence has been given, showing the number of rats in which that particular symptom was observed at the given dose. As a control four rats were treated with NaCl (100 mg/kg). The maximally effective doses for each symptom have been underlined.

As can be seen in Fig. 1, the dose which induced the maximal number of body shakes was, for MEC, 1.2 mmol/kg, whereas with doses higher than 1.8 mmol/kg a decrease was observed. With DMV, a maximal number of body shakes was obtained using 1.8 mmol/kg, while for DPA this was the case with 2.4 mmol/kg (Fig. 1A).

Maximum activity after injection of MEC was found using a dose of 0.9 mmol/kg, while maximal locomotor activity after DPA was obtained using 2.4 mmol/kg and after DMV, when using 3.0 mmol/kg. Caproate did not alter the activity, as compared with NaCl treatment, up to doses of 9.0 mmol/kg. The decrease in motor activity obtained after administration of high doses of the branched-chain fatty acids was caused by a loss of righting reflex occurring within the observation period (Fig. 1B).

As was found for body shakes and locomotor activity, so with regard to the total abstinence score (Fig. 1C), MEC was shown to be the most potent compound, eliciting a maximum score after administration of 0.9 mmol/kg. For DMV and DPA the highest abstinence score was evoked using a dose of 1.8 mmol/kg.

Comparing the magnitude of the maximal activity of the various compounds, DPA was found to be the most potent compound in evoking body shakes, horizontal activity and abstinence behaviour (Fig.1). Using MEC, a higher number of body shakes and activity counts was obtained when compared to DMV, whereas the DMV-induced maximal abstinence score was somewhat higher than found for MEC.

With regard to the checked signs (Table 1), no great differences were apparent between all compounds tested. Therefore, the differences in the total abstinence score can only be ascribed to differences in counted signs, such as body shakes and escape digging. As already mentioned, counted signs were not observed after injection of caproate.

Biochemistry

The kinetics of GABA-T were studied using concentrations of 0.5-5.0 mM GABA and 0.5 mM 2-oxoglutarate. The concentrations of MEC, DMV and caproate were 5, 10 and 20 mM for each compound. Lineweaver-Burke plots are presented in Fig. 2. From these plots the K_i's were calculated using a replot of the ratio of the K_m and V_{max} vs the concentration of the inhibitor and the calculated K_i's are presented in Table 2. MEC was found to be the most potent inhibitor, DMV was slightly less potent, whereas no inhibition could be found

Table 2.

Effects of DPA, MEC, DMV and Caproate on the activity of GABA-T and SSA-DH in rat brain homogenate

	GABA-T K_i (mM)	SSA-DH K_i (mM)	Ratio $\frac{K_i \text{ GABA-T}}{K_i \text{ SSA-DH}}$
DPA	23.2*	0.43	54
MEC	3.9	0.17	23
DMV	6.0	0.61	10
Caproate	NI	1.89	--

*$K_{i\ GABA-T}$ value from Van der Laan et al. (1979). Values were derived from the replots in the Figs. 2 and 3. The ratio is the quotient of the K_i for GABA-T and the K_i for SSA-DH of any one compound. NI = no inhibition.

with caproate. The inhibition by MEC and DMV had a mixed character, in which the competitive component was the greatest.

To study the effect of the fatty acids on SSA-DH, SSA concentrations of 50, 75 and 150 µM were used. In order to determine control values in the absence of an inhibitor, two additional concentrations were used, namely 25 and 100 µM. The concentrations of the fatty acids varied from 0.25 to 2.0 mM for MEC and from 0.5 to 4.0 mM for DPA, DMV and caproate, as can be seen in the replots (Fig. 3). For the determination of the K_m and V_{max} integrated Henri-Michaelis-Menten plots were used. The ratio of these parameters was plotted versus the concentration of the inhibitor to calculate the K_i. The resulting K_i's are presented in Table 2. With respect to SSA-DH, MEC was also found to be the most potent inhibitor, while the K_i of DMV was shown to be 1.5 times that found for DPA. The K_i for caproate was found to be 4.5 times that for DPA.

A relation between inhibition of GABA-T and inhibition of SSA-DH is given in Table 2, by expressing the inhibition of the GABA degradating enzymes as the ratio of the K_i's of these compounds.

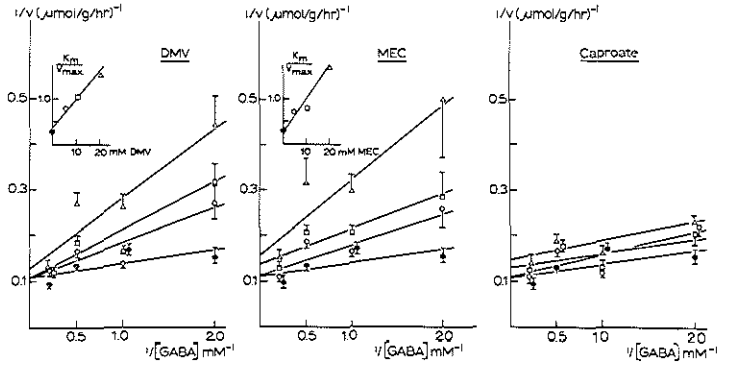

FIG. 2. The effect of DMV, MEC and Caproate on the activity of GABA-T. Reciprocal plots of the velocity vs the concentration of GABA in the presence of 0.5 mM 2-OG. For each concentration of the inhibitor the same symbol was used in all the figures: ●——●, control in the presence of 20 mM sodium-isethionate; o——o, 5 mM of a fatty acid; □——□, 10 mM of the fatty acid; Δ——Δ, 20 mM of the fatty acid. For DMV and MEC a replot of the ratios of the apparent K_m and V_{max} values vs the fatty acid concentration is inserted, to calculate the apparent K_i of these compounds. Bars represent SEM (n = 3).

DISCUSSION

Branched-chain fatty acids have been described as anaesthetic and neurodepressive agents (Lespagnol et al.,1971; 1972), while Marcus et al. (1967), using straight-chain fatty acids, reported an epileptoid rather than an anaesthetic state after administration of these fatty acids.

More recently, however, other behavioural effects of a branched-chain fatty acid, namely DPA, were reported using somewhat lower doses (Cowan and Watson, 1978; De Boer et al., 1977), e.g. body shakes and enhanced locomotor activity, besides other symptoms, resembling morphine abstinence behaviour. Therefore, this behaviour was presented as "quasi-morphine abstinence behaviour" (Bruinvels et al., 1980; De Boer et al., 1980) as defined by Collier (1974).

In contrast to the data of Lespagnol et al. (1972), these reports indicate a stimulating rather than a depressive action of this compound. The present

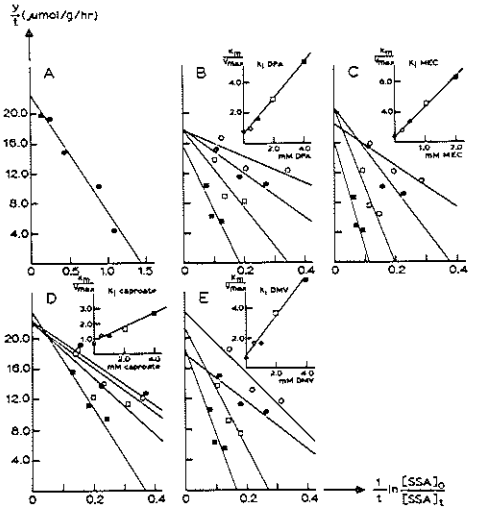

FIG. 3. The effect of DMV, DPA, MEC and Caproate on the activity of SSA-DH. Integrated Henri-Michaelis-Menten curves. Y is equal to the amount of product formed during the incubation time t. $[SSA]_o$ is the initial substrate concentration, whereas $[SSA]_t$ is the substrate concentration at the end of the incubation period. The slope of the lines obtained from these curves equals $-K_m$, while the intercept equals the apparent V_m. A: control in the presence of 10 mM sodium-isethionate. B: o——o, 0.5 mM DPA; ●——●, 1.0 mM DPA; □—— □, 2.0 mM DPA; ▩— ■, 4.0 mM DPA. C: o——o, 0.25 mM MEC; ●——●, 0.5 mM MEC; □——□, 1.0 mM MEC; ▩——■, 2.0 mM MEC. D: o——o, 0.5 mM Caproate; ●——●,1.0 mM Caproate; □—□, 2.0 mM Caproate; ▩——■, 4.0 mM Caproate. E: o——o, 0.5 mM DMV; ●——●, 1.0 mM DMV; □—□, 2.0 mM DMV; ▩——■, 4.0 mM DMV. Replots to determine the K_i's of the compounds are inserted, in which the ratio of the apparent K_m and V_{max} as a function of the inhibitor concentration is given. The results are the mean of three experiments.

results show that these behavioural effects could also be evoked after administration of two other branched-chain fatty acids, MEC and DMV.

Comparison of the doses of the compounds used for maximal induction of the behavioural syndrome revealed that MEC was more potent than DPA or DMV. The dose of MEC inducing the maximal score of body shakes, locomotor activity or total abstinence (1.0 mmol/kg-the mean of 1.2 for body shakes, 0.9 for horizontal activity and 0.9 for the total abstinence score) was found to be lower than those found for DPA or DMV (both 2.2 mmol/kg). On the other hand, the number of body shakes, the locomotor activity and the abstinence score were higher after injection of DPA at its maximally effective dose than after the maximally effective doses of DMV or MEC. After injection of DMV only a few body shakes could be shown, even at the maximally effective dose, whereas the scores for horizontal activity and abstinence were the same as for MEC.

The biochemical data obtained for MEC and DMV with regard to GABA-T are in agreement with those reported by Cash et al. (1975) and Maitre et al. (1978). However, other studies revealed a K_i for these compounds ten-fold lower than that obtained in the present study (Cunningham et al., 1980; Maitre et al., 1974). Possibly, this discrepancy may be due to the use of different methods. Although the K_i's for SSA-DH of the branched-chain fatty acids in the present experiments were eight-fold lower than those reported by Cash et al. (1975), the potency ranking was found to be the same. The K_i for DPA was shown to be the same as that reported previously (Van der Laan et al., 1979).

When considering the inhibition pattern of GABA-T by these compounds, it appeared that the compound with an intrinsic butyric acid chain, MEC, was the most potent inhibitor of GABA-T when compared to the compound with a propionic acid chain (DMV) or with a valeric acid chain (DPA); a conclusion which is in agreement with other reports (Fowler et al., 1975). When considering inhibition of SSA-DH, the length of the longest chain in the fatty acids studied seems to be more important, as has been reported previously for the straight-chain fatty acids (Bruinvels et al., 1980).

The present study clearly shows a great similarity in behavioural symptoms evoked by the branched-chain fatty acids, while the short straight-chain fatty acid, caproic acid, was inactive in this respect. Furthermore, the present results show that the three branched-chain fatty acids were acting as strong inhibitors of the degradation of GABA, especially by inhibition of SSA-DH.

Combining behavioural and biochemical data, a correlation appeared to exist between the maximally effective doses, evoking the strongest behavioural effects, and the K_i for SSA-DH of the various compounds studied. Thus, MEC, which elicits the behavioural syndrome at the low dose of 1.0 mmol/kg, has also been shown to be the strongest inhibitor of SSA-DH with a K_i of 0.17 mM. DPA and DMV, both exhibiting mean dose values evoking maximal behavioural effects higher than that found for MEC, namely 2.2 mmol/kg, are also less potent with regard to inhibition of SSA-DH with K_i values of 0.43 and 0.61 mM, respectively. The much higher K_i for SSA-DH or caproate (1.89 mM) may, therefore, indicate why no similar behavioural effects were observed with this fatty acid.

On the other hand, the number of body shakes, the increase in locomotor activity and the abstinence score appear to be dependent on the ratio of the K_i's for GABA-T and SSA-DH (Table 2). The high ratio (54) found for DPA seems to correlate with the highest behavioural score obtained with this compound, while the moderate ratio found for MEC (Sarhan and Seiler, 1979) and the low ratio found for DMV (Fowler et al., 1975) are in agreement with the moderate and low behavioural scores, respectively obtained with these compounds, especially with respect to body shakes.

These results may indicate that inhibition of SSA-DH by these branched-chain fatty acids, thus enhancing GABA levels, is responsible for the initiation of the quasi-morphine abstinence behaviour, a conclusion which is in agreement with the findings that the behaviour induced by DPA is antagonized by bicuculline, picrotoxin or 3-mercaptopropionic acid, but not by strychnine (De Boer et al., 1977, 1980). However, concurrent inhibition of GABA-T by these fatty acids -indicated by a lower ratio of the K_i's for both enzymes- results in a suppression of this abstinence behaviour. Intact GABA-T activity seems to be a requirement for full expression of the abstinence behaviour since pre-treatment of rats with the GABA-T inhibitor aminooxyacetic acid (AOAA) prevents DPA-induced behaviour (De Boer et al., 1980).

Since inhibition of either SSA-DH or GABA-T will both result in an increase in the GABA concentration, the results can be interpreted in favour of a compartmentalized action of DPA, suggesting a preferential inhibition of GABA-degradation, via SSA-DH, in the neuronal compartment as the action responsible for evoking quasi-abstinence behaviour, whereas an overflow of GABA to presynaptic areas, possibly GABA-ergic presynaptic receptors, might be responsible for suppression of this behaviour (Bruinvels et al., 1980; De Boer, 1977; De Boer et al., 1980).

A possible presynaptic action of GABA in inhibiting its own release has been suggested by several authors (Mitchell et al., 1978; Van der Heyden et al., 1980). Recently, Sarhan and Seiler (1979) presented evidence for a compartmentalized action of DPA in mouse brain, indicating an increase in synaptosomal GABA during the first 10 min and thereafter, an increase in non-synaptosomal material. This time course for the increase in synaptosomal GABA after DPA is in agreement with the time course of the quasi-abstinence behaviour induced by DPA.Iadarola and Gale (1979) have also provided evidence that the GABA-increase produced by DPA is associated mainly with GABA-ergic nerve terminals, while AOAA primarily elevates GABA in non-nerve terminal compartments.

Using the neuronal model as described above, MEC and DMV can be thought to inhibit the quasi-abstinence behaviour by enhancement of the concentration of non-synaptosomal GABA (probably located in glial cells and neuronal cell bodies). Release of GABA from the glial compartment will promote an action of GABA on presynaptic GABA-ergic autoreceptors resulting in inhibition of neuronal GABA-release. This explanation is supported by the recent findings of Cunningham et al. (1980), showing that MEC and DMV are capable of inhibiting GABA-dependent oxygen-uptake in non-synaptosomal mitochondria, while the very weak GABA-T inhibitor DPA was without effect in this system. Thus, DMV and MEC, but not DPA, inhibit GABA-metabolism in the non-synaptosomal compartment.

Although the role for GABA in true morphine-abstinence behaviour is still not clear, Ho et al. (1973, 1976) have shown that administration of AOAA or bicuculline can affect morphine analgesia, the development of tolerance and the degree of physical dependence.

Studies on the activity of glutamate decarboxylase during morphine dependency and abstinence (Ho and Gilliland, 1979; Tzeng and Ho, 1978) also indicate an enhanced synthesis of GABA (which is localized in the nerve endings) during the abstinence phase when compared to the dependency phase.

In conclusion, the present findings support a role for GABA in quasi-morphine abstinence behaviour evoked by branched-chain fatty acids. Inhibition of SSA-DH, resulting in an accumulation of GABA in the synaptosomal compartment, seems to be responsible for evoking quasi-morphine abstinence behaviour whereas inhibition of GABA-T, resulting in an increase in GABA-concentration in the non-synaptosomal compartment, will be responsible for suppression of this behaviour. The latter may occur via leakage or release of GABA from this compartment into the extraneuronal space, promoting an action of GABA on presynaptically located autoreceptors.

REFERENCES

Bruinvels, J., Van der Laan, J.W., De Boer, Th. (1980) GABA-degradation and its possible relationship to the "morphine abstinence"syndrome. Brain Res. Bull. 5, suppl. 2, 797-804

Cash, C., Cieselski, L., Maitre, M., Mandel, P. (1975) Purification de la semialdehyde succinique déshydrogénase de cerveau de rat et étude de son inhibition par des acides gras ramifiés. C.r. Séanc. Soc. Biol. 169, 884-887

Collier, H.O.J. (1974) The concept of the quasi-abstinence effect and its use in the investigation of dependence mechanisms. Pharmacology 11, 58-61

Cowan, A., Watson, T. (1978) Lysergic diethylamide antagonizes shaking induced in rats by five chemically different compounds. Psychopharmacology 57, 43-46

Cunningham, J., Clarke, D.D., Nicklas, W.J. (1980) Oxidative metabolism of 4-aminobutyrate by rat brain mitochondria: inhibition by branched-chain fatty acids. J. Neurochem. 34, 197-202

De Boer, Th.(1977)GABA shunt enzymes and the relationship with morphine abstinence. Thesis, Erasmus University, Rotterdam

De Boer, Th., Bruinvels, J. (1977) Assay and properties of 4-aminobutyric-2-oxoglutaric acid transaminase and succinic semialdehyde dehydrogenase in rat brain tissue. J. Neurochem. 28, 471-478

De Boer, Th, Metselaar, H.J., Bruinvels, J. (1977) Suppression of GABA-induced abstinence behavior in naive rats by morphine and bicuculline. Life Sci. 20, 933-942

De Boer, Th., Bartels, K., Metselaar, H.J., Bruinvels, J. (1980) Di-n-propylacetate induced abstinence behavior as a possible correlate of increased GABA-ergic activity in the rat. Psychopharmacology,71,257-267

Fowler, L.J., Beckford, J., John, R.A. (1975) An analysis of the kinetics of the inhibition of rabbit brain γ-aminobutyrate aminotransferase by sodium n-dipropylacetate and some other simple carboxylic acids. Biochem. Pharmac. 24, 1267-1270

Frederickson, R.C.A., Smits, S.E. (1973) Time course of dependence and tolerance development in rats treated with "slow release" morphine suspensions. Res. communs. chem.pathol. Pharmac. 5, 867-870

Harvey, P.K.P., Bradford, H.F., Davison, A.N. (1975) The inhibitory effect of sodium n-dipropylacetate on the degradative enzymes of the GABA-shunt. FEBS Lett. 52, 251-254.

Ho, I.K., Gilliland, T. (1979) Effects of acute and continuous morphine administration on the affinity of glutamic acid decarboxylase for pyridoxal 5-phosphate. Biochem. Pharmac. 28, 355-360

Ho, I.K., Loh, H.H., Way, E.L. (1973) Influence of GABA on morphine analgesia, tolerance and physical dependence. Proc. West. Pharmac. Soc. 16, 4-7

Ho, I.K., Loh, H.H., Way, E.L. (1976) Pharmacological manipulation of γ-aminobutyric acid (GABA) in morphine analgesia, tolerance and physical dependence. Life Sci. 18, 1111-1123

Iadarola, M.J., Gale, K. (1979) Dissociation between drug-induced increases in nerve terminal and non-nerve terminal pools of GABA *in vivo*. Eur. J. Pharmac. 59, 125-129

Lespagnol, A., Mercier, J., Erb-Debruyne, F., Dessaigne, S. (1971) Sur les propriétés pharmacodynamiques du méthyl-éthyl-caproate de sodium et en particulier son action anèsthétique générale de courte durée après injection intraveineuse. Therapie 26, 969-981

Lespagnol, A., Mercier, J., Erb-Debruyne, F., Dessaigne, S. (1972) Sur les propriétés neurodépressives et antiépileptiques d'acides organiques à chaîne ramifiée. Annls. pharm. fr. 30, 193-198

Marcus, R.J., Winters, W.D., Mori, K., Spooner, C.E. (1967) EEG and behavioral comparison of the effects of gamma-hydroxybutyrate, gamma-butyrolactone and short chain fatty acids in the rat. Int. J. Neuropharm. 6, 175-185

Maitre, M., Cieselski, L., Cash, C., Mandel, P. (1978) Comparison of the structural characteristics of the 4-amino-butyrate: 2-oxoglutarate transaminases from the rat and human brain, and their affinities for certain inhibitors. Biochim. Biophys. Acta 522, 385-399

Maitre, M., Cieselski, L., Mandel, P. (1974) Effect of 2-methyl, 2-ethyl caproic acid and 2,2-dimethyl valeric acid on audiogenic seizures and brain gamma aminobutyric acid. Biochem. Pharmac. 23, 2363-2368

Mitchell, P.R., Martin, I.L. (1978) Is GABA release modulated by presynaptic receptors? Nature 274, 904-906

Sarhan, S., Seiler, N. (1979) Metabolic inhibitors and subcellular distribution of GABA. J. Neurosci. Res. 4, 399-422

Segel, I.H. (1975) Enzyme Kinetics, John Wiley, New York

Tzeng, S.F., Ho, I.K. (1978) Acute and continuous morphine administration on the γ-aminobutyric acid system in the mouse. Prog. Neuro-Psychopharmac. 2, 55-64

Van der Heyden, J.A.M., Venema, K., Korf, J. (1980) *In vivo* release of endogenous GABA from rat striatum: effects of muscimol, oxotremorine and morphine. J. Neurochem. 34, 1648-1653

Van der Laan, J.W., De Boer, Th., Bruinvels, J. (1979) Di-n-propylacetate and GABA-degradation. Preferential inhibition of succinic semialdehyde dehydrogenase and indirect inhibition of GABA-transaminase. J. Neurochem. 32, 1769-1780

CHAPTER IV

A DUAL ROLE FOR GABA IN QUASI-MORPHINE ABSTINENCE BEHAVIOUR INDUCED BY DI-N-PROPYLACETATE INVOLVING BOTH INITIATION AND TERMINATION

J.W. van der Laan and J. Bruinvels

Psychopharmacology, in press

ABSTRACT

Di-n-propylacetate (DPA) induces in rats a behavioural syndrome resembling morphine abstinence behaviour. The inhibitory action of DPA on GABA degradation, resulting in an enhanced release of GABA, is probably responsible for this behavioural effect since GABA antagonists like bicuculline and picrotoxin have been shown to suppress this behaviour. However, the time course of the DPA induced behaviour is much shorter than that of the DPA induced enhancement of the GABA concentration. Therefore, we have studied the influence of enhanced GABA levels caused by a first injection of DPA and the behaviour evoked by a second injection of DPA at different time intervals after the first injection. The results indicate that GABA fulfils a role both in the initiation and termination of DPA induced behaviour.The mechanism responsible for this dual action of GABA is ascribed to a differential sensitivity to DPA of the nerve terminal and glial metabolic compartments of GABA in the brain. The increase of GABA in the nerve terminal caused by DPA is probably responsible for the initiation of the quasi-abstinence behaviour, whereas the overflow of GABA into the synaptic cleft may be responsible for the suppression of this behaviour via stimulation of pre-synaptic autoreceptors.

Another mechanism responsible for the rapid termination of the DPA evoked behaviour can be the formation of metabolites of DPA which may antagonize this behaviour. From experiments using some primary metabolites of DPA a role for these metabolites in the termination of the DPA induced behaviour seemed unlikely.

INTRODUCTION

It has been shown that administration of sodium-di-n-propylacetate (300 mg/kg i.p.; sodium valproate, Depakine^{R}, DPA) to naive rats induces a behavioural syndrome, including body shakes, resembling morphine abstinence behaviour (De Boer et al., 1977). This behaviour is antagonized by the GABA antagonists bicuculline and picrotoxin, but not by strychnine, a glycine antagonist, and a role for GABA in the expression of abstinence signs has been suggested (De Boer et al., 1977, 1980). In addition, it has been shown that DPA inhibits the degradation of GABA via inhibition of succinic semi-aldehyde dehydrogenase (Van der Laan et al., 1979). The behaviour induced by DPA in rats has, therefore, been suggested to represent a model for increased GABA-ergic activity (De Boer et al., 1980).

However, the time course of the increase in GABA concentration in rat brain after injection of DPA differs from the time course of the behaviour evoked by DPA. The latter reaches a peak value after 5 to 6 min and disappears after about 15 min (De Boer et al., 1977), while the maximum increase in GABA levels occurs 45 min after DPA administration and after 4 h has almost returned to normal (Kukino and Deguchi, 1977).

Thus, although the behaviour evoked by DPA appears to be initiated by an enhanced GABA-ergic activity, no correlation can be found between the time courses of behaviour and increased GABA. On the contrary, while the GABA concentration in brain is still increasing, the behaviour evoked by DPA has disappeared before the maximum GABA concentration has been achieved. To explain this paradox one can postulate that the behaviour is initiated by an action of DPA on the GABA-ergic nerve terminals -where DPA probably exerts its main action (Sarhan and Seiler, 1979; Iadarola and Gale, 1979)- by increasing the concentration of GABA and thus promoting the release of greater quantities of GABA. An overflow of GABA to presynaptic areas would subsequently stimulate the putative GABA-ergic autoreceptors, which may suppress the release of GABA and would thus terminate the DPA-evoked behaviour.

In the present study, directed towards the possible dual role of GABA in DPA-induced behaviour, namely in both initiation and termination of this behaviour, we have tested whether a second injection of DPA during the increase or during the decrease in the GABA concentration,caused by the first injection of DPA,would evoke the behaviour to the full extent.

Furthermore, the occurrence of tolerance to the behavioural effect was studied after repeated injections of DPA using longer time intervals, namely daily and weekly intervals. A possible development of tolerance over these time intervals is probably not related to enhanced GABA-levels, since 4 h and certainly 48 h after the DPA injection the GABA concentration has returned to normal (Kukino and Deguchi, 1977; Grimm et al., 1975).

Another possible mechanism responsible for the termination of the behaviour could be the rapid formation of one or more metabolites which do not evoke this behaviour themselves but may antagonize the behaviour induced by DPA. Therefore, three primary metabolites of DPA (Schäfer and Luhrs, 1978) were used to study this possibility.

The results obtained suggest that GABA indeed fulfils a dual role in the behaviour induced by DPA. No effect of the metabolites of DPA could be shown on the behaviour.

MATERIALS AND METHODS

Animals

Male albino rats (100-200 g) randomly selected from an inbred Wistar strain (TNO, Zeist, the Netherlands) were used in all experiments. The animals were housed four or five to a plastic cage with food and water ad lib. Lights were kept on from 8.30 a.m. till 8.30 p.m. The experiments were performed between 9.00 a.m. and 4.30 p.m. in a room with white noise and at a constant temperature of 22 $\pm$ 1°C.

Drugs

Sodium di-n-propylacetate (Sodium valproate, Albic BV, Maassluis, the Netherlands) was dissolved in deionized water (100 mg/ml) and administered intraperitoneally in a volume of 3 ml/kg (total dose 300 mg/kg), with the exception of experiment 2, where higher doses of DPA were used. Controls received an equimolar NaCl solution (33 mg/ml). The hydroxy-metabolites of DPA, sodium 3-hydroxy, 2-propylvalerate (3-OHDPA), sodium 5-hydroxy, 2-propylvalerate (5-OHDPA) and 4-hydroxy, 2-propylvalerolactone (4-OHDPA) were a gift from Dr. H. Schäfer (Desitin Werke Carl Klinke GmbH, Hamburg, FRG). The sodium salts of the metabolites were provided as a 10% solution in distilled water and were diluted to the appropriate volume with deionized water, whereas the lactone was dissolved in an equimolar quantity of sodium hydroxide to hydrolyse the lactone.

Behaviour

Observation of the behaviour was carried out essentially as described by De Boer et al. (1977). Briefly, rats were allowed to adjust to a cage with sawdust on the bottom for at least 30 min, whereafter the drug or salt solution was administered intraperitoneally. Subsequently, the behaviour was observed for 15 min. Scores of 0 (absent), 2 (mild) or 4 (marked) were given to penile erection, penis licking, teeth chattering, swallowing, tremor, hunchback posture and piloerection. Escape digging, body shakes and foreleg shakes were counted and assigned scores of 2, 4, 6, 8 or 10 if they occurred 2-5, 6-10, 11-20, 21-40 or more than 40 times, respectively, during the observation period of 15 min. Other signs characteristic of the morphine abstinence syndrome, as indicated by Frederickson and Smits (1973) were not seen in DPA-treated animals.

Activity measurements

Horizontal and vertical activity was measured using a Varimex activity meter as described by De Boer et al. (1977).

Statistics

The Mann-Whitney U-test, the Wilcoxon paired signed rank test and Fisher's exact probability test (Siegel, 1956) were used for evaluating the results as indicated in the legends to the tables and figures. Differences with a probability equal to or lower than 5% were considered to be statistically significant.

PROCEDURES

Experiment 1

Rats were treated with DPA (300 mg/kg i.p.) and their behaviour was observed. After 20 min, 2 h or 4 h the rats received a second injection of DPA (300 mg/kg i.p.), whereafter behaviour was scored again for 15 min. Controls received as a first or a second treatment an injection of NaCl (100 mg/kg i.p.).

Experiment 2

Naive rats received graded doses of DPA, i.e. 300, 400, 600 or 900 mg/kg i.p., whereafter their behaviour was scored for 15 min. The observation period was divided into three subperiods of five minutes.

Experiment 3

Rats received daily or weekly injections of DPA (300 mg/kg i.p.), whereafter their behaviour was scored for 15 min. Controls received NaCl (100 mg/kg i.p.). The exact injection scheme is presented in Table 1.

Table 1.

Scheme for the treatment of the animals in the study of daily or weekly tolerance

	Day							
Group	1	2	3	4	7	14	21	
1 (11)	D	D	D	D				3A
2 (11)	D				D	D	D	3B
3 (6)					D	D	D	
4 (6)						D	D	
5 (6)							D	

D = DPA 300 mg/kg i.p.
The figure in which the results are presented is indicated on the right.
Numbers between brackets represent the number of animals.

Experiment 4

3-OHDPA (30 mg/kg), 4-OHDPA (15 mg/kg), 5-OHDPA (15 mg/kg) and saline (3 ml/kg) were administered intraperitoneally.

Since Cieselski et al. (1975) reported that the maximum fixation of DPA in rat brain was achieved 30-40 min after intraperitoneal injection, a pretreatment-time of 40 min was chosen for the hydroxy metabolites of DPA. Thus, 40 min after injection of these compounds the rats received an injection of DPA (300 mg/kg) or NaCl (100 mg/kg), whereafter their behaviour was scored for 15 min.

RESULTS

Experiment 1: Acute tolerance study

The data in Table 2 indicate that a second injection of DPA administered 20 min after the first injection could not evoke the full abstinence syndrome. The total abstinence score and the number of body shakes were decreased by 48 and 56%, respectively. The decrease in the total abstinence score was not only due to a decrease in the number of body shakes, but also to a lower score of escape digging, a decreased incidence of penis licking and a tendency towards a decrease in the incidence of ptosis and other signs, mainly swallowing and head shakes.

Table 2. Acute tolerance to the behavioural effect of DPA

A	abstinence score	body shakes	horizontal activity	vertical activity
1st injection (35)	100%	100%	100%	100%
2nd injection after:				
20 min (10)	52.1 ± 5.9**	43.5 ± 6.7**	109.7 ± 6.5	105.7 ± 12.0
2 h (9)	76.6 ± 11.6 *	56.4 ± 6.7**	65.8 ± 6.9**	63.8 ± 7.9**
4 h (10)	85.4 ± 7.8	80.5 ± 11.4*	70.8 ± 6.4**	64.4 ± 5.9**
24 h (9)	116.2 ± 6.4	89.4 ± 8.6	101.6 ± 9.0	94.9 ± 13.5

B	hunchback posture	piloerection	escape digging	ptosis	penis licking	other signs
1st injection (35)	100	76	63	38	54	1.8
2nd injection after:						
20 min (10)	90	70	20**	20	0**	0.6
2 h (9)	100	90	44	90*	30	1.0
4 h (10)	100	100	10**	80	10	1.4
24 h (9)	100	100	78	67	78	1.3

Animals were treated twice with DPA (300 mg/kg) at different time intervals. Behaviour was observed for 15 min. Values are given as means ± SEM.

A. The values given for body shakes, abstinence score, horizontal and vertical activity are expressed as the percentage of the behavioural parameter obtained after the first injection of DPA.
*, $p \leq 0.05$; *, $p \leq 0.01$, statistical significance vs the first injection of DPA (Wilcoxon, two tailed).

B. The values given for hunchback posture, piloerection, escape digging, ptosis and penis licking represent incidence ratios in percentages. The item "other signs" include swallowing, penile erection, head shakes. The value given represents the contribution of these signs to the total abstinence score, given in A, according to Frederickson and Smits (1973).
*, $p \leq 0.01$; ** , $p \leq 0.002$, statistical significance tested vs the first injection of DPA (Fisher, one tailed).

Numbers between brackets represent the number of observations.
The values given for 24 h are taken from experiment 3.

After this second injection of DPA, no difference was found in motor activity. DPA administration 2 h after the first injection still yielded an impaired abstinence score, but to a lesser extent than that obtained with DPA administration 20 min after the first injection, due to an enhanced incidence of ptosis and the return of penis licking. Horizontal and vertical activity was also suppressed by about 35% when the second DPA injection was given 2 h after the first administration. Injection of DPA 4 h after the first injection yielded an abstinence score which did not differ significantly from that obtained after the first injection, although the number of body shakes was still suppressed by 20%. The locomotor activity was decreased by about 30% when compared with the activity after the first injection of DPA. When the interval between the first and the second injection of DPA was 24 h (results obtained from experiment 3), no significant change in any of the behavioural parameters studied could be found.

The incidence of escape digging was found to be decreased significantly when DPA was administered 20 min and 4 h after the first administration.

Experiment 2: Response after high doses of DPA

From Fig. 1 it can be seen that the maximal effective dose of DPA was 400 mg/kg, both for body shakes and locomotor activity. The main difference between the behavioural responses obtained after administration of the various doses of DPA is the shift of the maximal activity with time. Using doses of 300 and 400 mg/kg the highest locomotor activity was found during the second 5 min period, whereas with 900 mg/kg the peak was found during the first period. No differences could be shown between the scores obtained after administration of 300 and 600 mg/kg during the total period of 15 min. For all doses, except 400 mg/kg, a significant difference between the number of body shakes was found, when comparing the first and second periods of 5 min. However, for 300 mg/kg the score for the second observation period was higher, whereas for 600 and 900 mg/kg the highest score was obtained during the first period.

Escape digging, swallowing and teeth chattering were only seen when using doses of 300 and 400 mg/kg and were absent after the use of higher doses of DPA. Hunchback posture and piloerection occurred in all rats during the first 5 min period after DPA administration. However, using doses of 600 and 900 mg/kg of DPA a lower incidence of these latter symptoms was

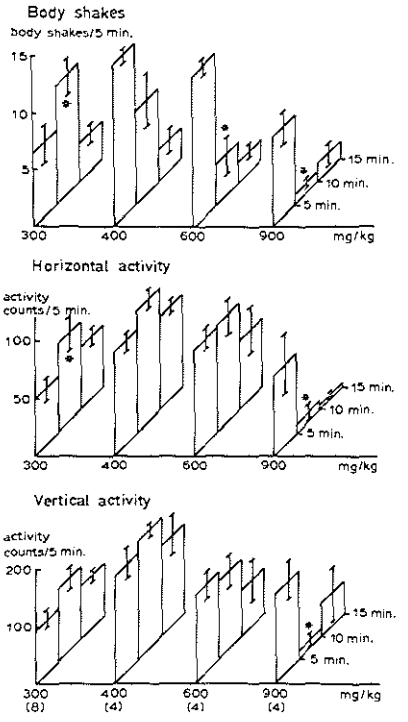

FIG. 1. Effect of increasing doses of DPA on body shakes and activity. Rats were treated with different doses of DPA whereafter behaviour was observed for 15 min. Abscissa: different doses of DPA. Oblique abscissa: subdivision of the 15 min after injection of DPA into three 5 min periods. Numbers between brackets represent number of animals. Bars represent SEM, *, $p < 0.05$ vs the first period of 5 min (Mann Whitney-U., two tailed).

observed during the second and third observation period (data not shown).

Experiment 3: subchronic tolerance study

As can be seen in Fig. 2A, daily injections of DPA resulted in tolerance to body shakes, escape digging and motor activity.

After the third and fourth injection the number of body shakes was reduced to 50-60%. Escape digging and locomotor activity were decreased to 40% and 80%, respectively, after the third and fourth DPA injection. These differences, however, were statistically significant only after the fourth injection.

Development of tolerance could also be observed for body shakes and locomotor activity if intervals of a week instead of a day were used (Fig. 2B). The decrease resulting in the most significant difference was also found after the second injection of DPA, whereas subsequent administration of DPA did not cause a further decrease.

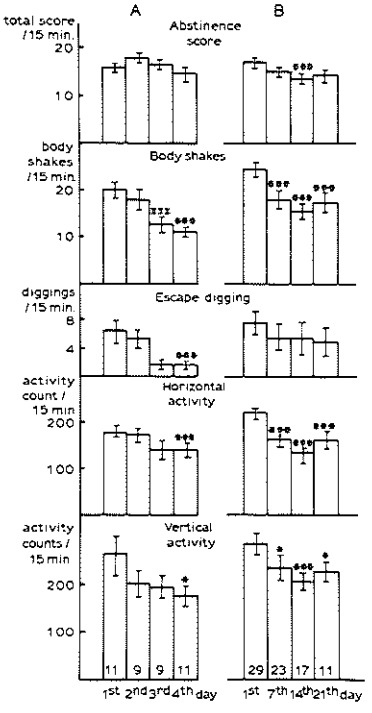

FIG. 2. Tolerance to the behavioural effects of DPA after daily and weekly injection.
A. Daily injections of DPA (300 mg/kg i.p.). Behaviour was observed for 15 min. The effects of each of four daily injections were scored.
B. Weekly injections of DPA (300 mg/kg i.p.). Rats were treated with a maximum of four injections over a total period of three weeks.
*, $p \leq 0.05$; * *, $p \leq 0.02$; * * * $p \leq 0.01$, statistical significance tested vs the first DPA injection (Wilcoxon, two tailed).
The numbers at the base of the columns for the vertical activity represent the number of observations. Bars represent SEM.

Experiment 4: Effect of hydroxy-metabolites

None of the three hydroxy-metabolites of DPA, given in doses of 30 mg/kg for 3-OHDPA and 15 mg/kg for 4-OHDPA and 5-OHDPA, affected the behaviour evoked by DPA (Table 3), nor did they induce abstinence behaviour themselves. Higher doses (300 mg/kg; data not shown) were also without effect.

Table 3. Behavioural effect of DPA after pretreatment with hydroxy metabolites

Item	Saline (6)	3-OHDPA (6)	4-OHDPA (6)	5-OHDPA (5)
Abst. score	19.7 ± 1.6	16.7 ± 2.0	16.0 ± 2.7	22.8 ± 1.9
Body shakes	30.7 ± 5.0	25.0 ± 4.8	25.0 ± 3.0	32.8 ± 5.7
Head shakes	1.7 ± 0.8	0.8 ± 0.5	1.0 ± 0.3	3.0 ± 0.9
Escape digging	9.8 ± 1.8	9.5 ± 4.5	7.5 ± 4.0	11.4 ± 2.7
Horizontal activity	202.3 ± 14.6	198.5 ± 18.9	214.2 ± 10.4	219.6 ± 38.5
Vertical activity	357.7 ± 18.6	348.7 ± 29.5	321.7 ± 31.3	380.0 ± 36.5
Ptosis	0/6	2/6	1/6	2/5
Penis licking	3/6	4/6	0/6	2/5
Swallowing	4/6	3/6	1/6	2/5
Hunchback posture	6/6	6/6	6/6	5/5
Piloerection	6/6	6/6	6/6	5/5

Effect of pretreatment with hydroxy metabolites of DPA on the behavioural response to DPA. Rats were treated with 3-OHDPA (30 mg/kg), 4-OHDPA (15 mg/kg), 5-OHDPA (15 mg/kg) or saline and received an injection with DPA (300 mg/kg) after 40 min following which their behaviour was observed for 15 min. Values for the abstinence score, the counted signs and the activity were given as means ± SEM. Values for the checked signs represent the incidence. Numbers between brackets are the number of animals.

DISCUSSION

It has been shown previously that the behaviour evoked by the administration of DPA (300 mg/kg) resembles morphine abstinence behaviour (De Boer et al., 1977). In addition, it has been reported that the DPA-induced behaviour may represent a correlate of increased GABA-ergic activity (De Boer et al., 1977, 1980). However, the time course of this behaviour is different from the time course of the increase in GABA concentration in brain. While rats showed maximal induction of behaviour 5-6 min after DPA injection, this behaviour disappeared after about 15 min. However, the level of GABA has been reported to increase until 30-45 min after the administration of DPA. Thereafter, GABA concentration remains constant for more than one hour and then decreases to nearly normal GABA levels during the next 2 h (Kukino and Deguchi, 1977). Since the DPA evoked behaviour could be inhibited by the administration of bicuculline or picrotoxin (De Boer et al., 1977, 1980) it has been concluded that the behaviour is mediated by an increased

GABA-ergic stimulation. However, the short duration of the behaviour as compared to the increase in brain GABA level does not seem to favour a correlation between those two phenomena. In the present paper the effect of pretreatment with DPA itself, which will cause an increase in brain GABA concentration, has been studied on the DPA induced behaviour evoked by a second injection of DPA.

When rats, pretreated with DPA, received a second injection of DPA shortly after the first injection, the latter could not evoke the behaviour to the same extent as that obtained after the first DPA administration. The strongest suppression of body shakes and total abstinence score could be observed if a second injection of DPA was given 20 min or 2 h after the first DPA administration. However, if DPA was administered 4 h after the first injection, the behavioural scores obtained were still somewhat impaired, but only the number of body shakes differed significantly from that obtained after the first DPA injection. The reason that this lower number of body shakes is not reflected in a lower abstinence score lies with the method of calculation of the abstinence score, i.e. there is a certain range in the number of body shakes to which the same score will be given for calculation of the abstinence score (see under Materials and Methods).

When the time course of the behavioural suppression is compared with the known time course of the increase in GABA concentration after a single injection of DPA (Kukino and Deguchi, 1977; *vide supra*), it rather suggests that suppression of the DPA evoked behaviour and not the behaviour itself was induced by an enhanced GABA level. This suppression is already maximal during the increase in the brain GABA concentration and is still present 2 h after the DPA injection, when the brain GABA level has achieved maximum. When, however, the level of GABA is decreased at 4 h after administration of DPA, the abstinence behaviour can be evoked by a second injection of DPA to nearly the full extent. Therefore, the results strongly suggest a relationship between inhibition of DPA-evoked behaviour and GABA concentration in the brain. In contrast, the decrease in locomotor activity, which was found to have a different time course when compared with the body shakes (Table 2), is probably caused by a mechanism secondary to the enhanced GABA level.

That an increase in the GABA concentration will suppress DPA-induced behaviour is supported by the finding that pretreatment with low doses of the GABA-T inhibitor amino-oxyacetic acid (AOAA) prevents the induction of this behaviour (De Boer et al., 1980).

The question arises as to the mechanism of the DPA-induced enhancement of GABA, since on the one hand it seems obvious that enhancement of GABA-ergic stimulation is involved in the DPA-evoked behaviour, while, on the other hand GABA may play a role in suppression of this behaviour.

It is well-known that the metabolism of GABA is compartmentalized (Balazs et al., 1972). It may be that these compartments are associated with certain functionally distinguishable structures in the brain, such as nerve endings, glial cells and neuronal cell bodies (for a review see Hertz, 1979). To explain the fact that pretreatment of rats with AOAA prevents the induction of abstinence behaviour by DPA, it has been suggested that accumulation of GABA by AOAA will result in leaking of GABA from glial cells reaching presynaptic autoreceptors, thus inhibiting the enhanced release of GABA induced by DPA (De Boer, 1977; De Boer et al., 1980). Recently, a different localization for the GABA-enhancing action of DPA and AOAA has been reported (Iadarola and Gale, 1979), indicating that DPA only exerts its action in the neuronal compartment, while AOAA acts in both the glial and the neuronal compartment.

The present findings, therefore, support the postulated model in which the DPA-induced increase in nerve terminal GABA results in an increased release of GABA into the synaptic cleft and an activation of a post-synaptically localized receptor. Subsequently, a further increase in the GABA concentration may activate the GABA-ergic autoreceptors resulting in an inhibition of the enhanced GABA release. Regulation of GABA release via stimulation of presynaptic autoreceptors has been reported (Mitchell and Martin, 1978; Van der Heyden et al., 1980). This mechanism, involving an overflow of GABA has been supported recently by Sarhan and Seiler (1979) who described an initial rise of GABA induced by DPA during the first 10 min after its administration in synaptosomal fractions only, whereas after this period an increase in non-synaptosomal fractions is observed.

It has been reported that the DPA-induced enhancement of the GABA concentration is dose-dependent (Simler et al., 1978), but that the time to achieve the maximal increase appears to be the same at all doses. This indicates that a higher dose of DPA will result in a more rapid increase in GABA levels. Therefore, different doses of DPA were used to study the effect of this more rapid enhancement on the time course of the behavioural syndrome.

The results obtained are in agreement with the dual role of GABA in the behaviour as discussed above. On the one hand, a more rapid increase in GABA

will result in a shorter time of onset of the behaviour and a higher initial score, while on the other hand, due to the faster increase in GABA concentration, the overflow of GABA to GABA-ergic autoreceptors will occur earlier after the injection of DPA.

Alternatively, the present data might be explained assuming different functions for GABA-ergic systems, which are localized in different areas of the brain viz. an inhibitory function e.g. in the nucleus accumbens (Scheel-Krüger et al., 1978) and a disinhibitory one e.g. in the ventral tegmental area or in the substantia nigra (Arnt and Scheel-Krüger, 1979; Scheel-Krüger, 1978). In this hypothesis DPA would enhance GABA-ergic transmission at first in excitatory areas, while secondary an increase in the GABA concentration in other areas will result in inhibition. However, the finding that treatment of rats with a low dose of AOAA did not result in a similar syndrome as that observed after DPA and even suppressed the latter (De Boer et al., 1980) would favour an interpretation involving autoreceptors as discussed above.

The results presented in Fig. 2 indicate the induction of tolerance after both daily and weekly injections of DPA. The degree of tolerance development is much lower, especially after weekly injections, than has been observed when a second injection of DPA was given within 2 h after the first DPA injection (Table 2). This indicates that probably different systems are responsible for the observed tolerance using this different schedule. In addition, Grimm et al. (1975) have shown that the concentration of GABA has been returned to normal levels 48 h after administration of DPA.

Although there are strong indications for a role of GABA in the termination of the DPA-induced behavioural syndrome, other mechanisms cannot be ruled out. Another possible mechanism might be the rapid impairment of DPA-induced behaviour by one or more metabolites of DPA which themselves do not induce such behaviour but suppress the behaviour induced by DPA. The tolerance evoked by daily or weekly administration of DPA might be explained then by an enhanced formation of metabolites, e.g. by enzyme induction.

To test whether inhibition of the behaviour by metabolites of DPA could occur, we used the initial metabolites of DPA, namely 3-OHDPA which is mainly formed in rats (Jakobs and Löscher, 1978). In view of the studies on the levels of DPA metabolites found in rat urine after 24 h (Kukino et al., 1972; Kochen et al., 1977; Ferrandes and Eymard, 1977), doses of 30 mg/kg 3-OHDPA, 15 mg/kg 4-OHDPA and 5-OHDPA were used to test this possibility as stated above.

Since no inhibitory effect of these metabolites was observed (Table 3), it seems unlikely that the metabolites of DPA play a role in the termination of the behaviour or in the occurrence of acute tolerance. However, a role for a metabolite of DPA which was not tested (e.g. 2-propyl-glutaric acid; Kuhara and Matsumoto, 1974) cannot be excluded in the present experiments.

The DPA-induced behaviour can also be considered as a model for morphine abstinence behaviour (Bruinvels et al., 1980) and might be important in view of the short time course of the behaviour. This short time course is in agreement with that observed after precipitation of abstinence behaviour with naloxone in opiate-dependent animals. In this case the maximal behavioural score was found within 10 min after naloxone injection and the behaviour returned to normal after 20-30 min (Bläsig et al., 1973). This similarity in time course between naloxone-precipitated abstinence and DPA-induced quasi-abstinence behaviour may suggest that similar processes, i.e. a dual role for GABA both in initiation and termination, may also be involved in naloxone-precipitated abstinence. More direct evidence for the involvement of opiate sensitive systems in DPA-induced behaviour can be derived from the observed cross-tolerance between DPA and RX 336-M, a codeine derivative, for the induction of body shakes (Cowan and Watson, 1978).

In conclusion, the present results suggest that GABA may play a role not only in the initiation of the DPA-induced behaviour but also in its termination. An explanation for the dual role of this neurotransmitter is provided by the differential action of DPA on the distinct metabolic compartments of GABA. According to this mechanism, DPA preferentially acts on the neuronal system, thus initiating abstinence behaviour, whereas the overflow of GABA to presynaptic areas may activate presynaptic autoreceptors thus via inhibition of the presynaptic release of GABA terminating the GABA-evoked abstinence behaviour.

REFERENCES

Arnt, J., Scheel-Krüger, J. (1979) GABA in the ventral tegmental area: differential regional effects on locomotion, aggression and food intake after micro-injection of GABA agonists and antagonists. Life Sci. 25, 1351-1360

Balazs, R., Machiyama, Y., Patel, A.J. (1972) Compartmentation and the metabolism of γ-aminobutyrate. In: Metabolic compartmentation in the brain. R. Balazs and J.E. Cremer, eds. Macmillan, London pp. 57-70

Bläsig, J., Herz, A., Reinhold, K., Zieglgänsberger, S. (1973) Development

of physical dependence on morphine in respect to time and dosage and quantification of the precipitated withdrawal syndrome in rats. Psychopharmacol. 33, 19-38

Bruinvels, J., Van der Laan, J.W., De Boer, Th. (1980) GABA degradation and its possible relationship to the "morphine abstinence" syndrome. Brain Res. Bull. 5, suppl. 2, 797-804

Cieselski, L., Maitre, M., Cash, C., Mandel, P. (1975) Regional distribution in brain and effect on cerebral mitochondrial respiration of the anti-convulsive drug n-dipropylacetate. Biochem. Pharmac. 24, 1055-1058

Cowan, A., Watson, T. (1978) Lysergic acid di-ethylamide antagonizes shaking induced in rats by five chemically different compounds. Psychopharmacol. 57, 43-46

De Boer, Th. (1977) GABA shunt enzymes and the relationship with morphine abstinence. Ph.D. Thesis, Erasmus University, Rotterdam

De Boer, Th., Metselaar, H.J., Bruinvels, J. (1977) Suppression of GABA-induced abstinence behaviour in naive rats by morphine and bicuculline. Life Sci. 20, 933-942

De Boer, Th., Bartels, K., Metselaar, H.J., Bruinvels, J. (1980) Di-n-propylacetate induced abstinence behaviour as a possible correlate of increased GABA-ergic activity in the rat. Psychopharmacol. 71, 257-267

Ferrandes, B., Eymard, P. (1977) Metabolism of Valproate sodium in rabbit, rat, dog and man. Epilepsia 18, 169-182

Frederickson, R.C.A., Smits, S.E. (1973) Time course of dependence and tolerance development in rats treated with "slow release" morphine suspensions. Res. Commun. Chem. Pathol. Pharmacol. 5, 867-870

Grimm, V., Gottesfeld, Z., Wasserman, I., Samuel, D. (1975) The level of GABA in the brain and locomotor behaviour. Pharmac. Biochem. Behav. 3, 573-578

Hertz, L. (1979) Functional interactions between neurons and astrocytes I. Turnover and metabolism of putative amino-acid transmitters. Prog. Neurobiol. 13, 277-323

Iadarola, M.J., Gale, K. (1979) Dissociation between drug-induced increases in nerve terminals and non-nerve terminal pools of GABA *in vivo*. Eur. J. Pharmacol. 59, 125-129

Jakobs, C., Löscher, W. (1978) Identification of metabolites of valproic acid in serum of humans, dog, rat en mouse. Epilepsia 19, 591-602

Kochen, W., Imbeck, H., Jakobs, C. (1977) Studies in the urinary excretion of metabolites of valproic acid (dipropylacetic acid) in rats and humans. Arzneim.-Forsch./Drug Res. 27, 1090-1099

Kukino, K., Deguchi, T. (1977) Effects of sodium dipropylacetate on γ-amino-butyric acid and biogenic amines in rat brain. Chem. Pharm. Bull. 25, 2257-2262

Kukino, K., Mineura, K., Deguchi, T., Ishii, A., Takahira, H. (1972) Studies on a new anticonvulsant drug, sodium dipropylacetate. Assay for metabolites and metabolic pathway. J. Pharm. Soc. Jap. 92, 896-900

Kuhara, T., Matsumoto, I. (1974) Metabolism of branched medium chain length fatty acid I. ω-Oxidation of sodium dipropylacetate in rats. Biomed. Mass Spectrom. 1, 291-294

Mitchell, P.R., Martin, I.L. (1978) Is GABA release modulated by pre-synaptic receptors? Nature 274, 904-905

Sarhan, S., Seiler, N. (1979) Metabolic inhibitors and subcellular distribution of GABA. J. Neuroscience Res. 4, 399-422

Schäfer, H., Luhrs, R. (1978) Metabolite pattern of valproic acid. Part 1: Gaschromatographic determination of the valproic acid metabolite

artefacts, heptanone-3, 4- and 5-hydroxyvalproic acid lactone. Arzneim. Forsch./Drug Res. 28, 657-662
Scheel-Krüger, J., Arnt, J., Braestrup, C., Christensen, A.V., Cools, A.R., Magelund, G. (1978) GABA-dopamine interaction in the substantia nigra and nucleus accumbens -relevance to behavioural stimulation and stereotyped behaviour. In: Dopamine, P.J. Roberts, G.N. Woodruff, L.L. Iversen, eds. Advances in Biochemical Psychopharmacology, Vol. 19, Raven, New York pp. 343-346
Siegel, S. (1956) Non-parametric statistics for the behavioural sciences. McGraw Hill, Kogakusha, Tokyo
Simler, S., Gensburger, C., Cieselski, L. Mandel, P. (1978) Time course of increase in GABA level in different mice brain regions following n-dipropylacetate treatment. Commun. Psychopharmacol. 2, 123-130
Van der Heyden, J.A.M., Venema, K., Korf, J. (1980) In vivo release of endogenous GABA from rat striatum: effects of muscimol, oxotremorine and morphine. J. Neurochem. 34, 1648-1653
Van der Laan, J.W., De Boer, Th., Bruinvels, J. (1979) Di-n-propyl-acetate and GABA degradation. Preferential inhibition of succinic semialdehyde dehydrogenase and indirect inhibition of GABA-transaminase. J. Neurochem. 32, 1769-1780

CHAPTER V

DIPROPYLACETATE-INDUCED QUASI-MORPHINE ABSTINENCE BEHAVIOUR IN THE RAT: INVOLVEMENT OF AMYGDALOID AND THALAMIC STRUCTURES

J.W. van der Laan and J. Bruinvels

Brain Research, accepted for publication

ABSTRACT

Quasi-morphine abstinence behaviour induced by di-n-propylacetate (DPA) in rats is thought to be caused by an increased GABA-ergic activity in the CNS.

Behavioural responses after intracerebral injections of DPA were studied to gain insight into the brain sites involved in this quasi-abstinence syndrome. Injection of DPA into the centre median-parafascicularis (Cm-Pf) resulted in a large number of body shakes and greater locomotor activity when compared to other brain areas. Injection of DPA into the central amygdala (Ac) resulted in an enhanced number of chewing episodes.

Administration of bicuculline methiodide (BMI) into the Cm-Pf, 5 min after intraperitoneal administration of DPA, suppressed body shakes but had not only minor effects on horizontal activity, whereas injection of morphine into the same structure suppressed both behavioural symptoms. It is concluded that GABA-ergic and opioid mechanisms in the Cm-Pf are involved in the DPA-induced behaviour.

Injection of BMI into the central amygdala shortly after i.p. injection of DPA resulted in an increase in the number of body shakes, whereas no effect was observed on activity. Morphine applied to this structure potentiated slightly the locomotor activity, but had no effect on the body shakes induced by DPA.

The present results suggest a facilitatory role for a GABA-ergic system in the Cm-Pf on body shakes, while in the central amygdala a GABA-ergic system exerts an inhibitory influence on this symptom of abstinence.

INTRODUCTION

Di-n-propylacetate (DPA, Depakine^{R}) induces a specific syndrome resembling morphine abstinence behaviour in rats who have never been exposed to opiates (De Boer et al., 1977). The syndrome mainly involves body shakes, escape digging, hunchback posture, piloerection and enhanced locomotor activity. Non-sedative

doses of morphine suppress this behaviour, indicating a quasi-abstinence type of behaviour (De Boer et al., 1980). Since the DPA-induced abstinence behaviour can be antagonized by bicuculline or picrotoxin, but not by strychnine, increased GABA-ergic activity is the mechanism held responsible for the syndrome (De Boer et al., 1977, 1980). DPA increases GABA-ergic activity via inhibition of the second enzyme involved in GABA degradation, i.e. succinic semialdehyde dehydrogenase (SSA-DH) (Van der Laan et al., 1979) and not via a direct effect on the first enzyme, GABA-transaminase (GABA-T), as suggested by others (Godin et al., 1969; Simler et al., 1973). Inhibition of SSA-DH results in accumulation of SSA, which inhibits GABA-T, thus increasing the concentration of GABA (Van der Laan et al., 1979). Recent studies with branched-chain fatty acid analogues of DPA support this hypothesis, revealing a correlation between inhibitory effects on SSA-DH and the behavioural effects of these compounds (Van der Laan et al., 1980).

The present experiments were designed to investigate the role of brain structures involved in the expression of these quasi-morphine abstinence signs evoked by DPA and also to gain a better insight into the neuro-anatomical pathways involved in the morphine withdrawal syndrome. The latter pathways have been analyzed thus far by intracerebral application of naloxone in opiate-dependent animals (Calvino et al., 1979; Lagowska et al., 1978; Laschka and Herz, 1977; Wei et al., 1972, 1973, 1975), or by lesioning, electrolytically or chemically, of brain pathways, whereafter abstinence behaviour was precipitated by systemic administration of naloxone (Calvino et et al., 1979; Elchisak and Rosecrans, 1979; Kerr and Pozuelo, 1971). Intracerebral injection of DPA might reveal a relationship between specific GABA-ergic systems and abstinence behaviour.

In the present experiments DPA was injected into several structures which are known to contain GABA-ergic receptors, e.g. the caudate nucleus, the substantia nigra, hippocampus and amygdala and quasi-morphine abstinence behaviour was observed. Only behavioural responses elicited in the central amygdala (Ac) and the centre median-parafascicularis area (Cm-Pf) correspond to the quasi-abstinence syndrome and were further characterized. Both the Ac and the Cm-Pf are known to contain a moderate to high density of opiate receptors (Atweh and Kuhar, 1977 a,b).

MATERIALS AND METHODS

Ninety-four male albino rats (180-220 g, at the time of surgery), randomly selected from an inbred Wistar strain (TNO, Zeist, The Netherlands) were used. The animals were housed in groups of four or five in Macrolon cages (47x27x 15 cm) and received food and water ad lib. Lights were kept on from 8.30 to 20.30 h.

Animals (n=66) were anaesthetized with chloralhydrate (400 mg/kg i.p.) for stereotaxic surgery and double barrelled stainless steel cannulae were implanted bilaterally. The coordinates, according to the Atlas of König and Klippel (1963), are presented in Table 1. For the centre median, the cannulae were placed at an angle of 6^{o}. The cannulae were inserted 6.0 mm below the skull surface for the amygdala and 4.0 mm for the centre median. After placement the cannulae were fixed to the skull with acrylic dental cement (Paladur^{R}). After a recovery period of one week, during which the animals were housed individually, the animals received a bilateral injection of 10 µg DPA in 0.5 µl artificial CSF (Palaic et al., 1967), using a 10 µl Hamilton syringe (31 gauge needle).

Rats were allowed to habituate to a cage with sawdust bedding for at least 30 min. Directly after the intracerebral injection, behaviour was observed for 15 min. The following signs were scored: hunchback posture, piloerection, ptosis and penile erection. Other signs were recorded as quantal events: rearing, foreleg shakes, head shakes, body shakes, escape digging and episodes of chewing, swallowing and teeth chattering. Locomotor activity was monitored over the 15 min. observation period using a Varimex activity meter. Behaviour was observed between 10.00 and 16.30 h in a room with white noise and a constant temperature of $22 \pm 1^{o}C$.

Four to seven days after the intracerebral injection of DPA, the animals received an intraperitoneal injection of DPA (300 mg/kg) after which their behaviour was observed, as described above. After five minutes bicuculline-methiodide (BMI), strychnine or vehicle was injected intracerebrally.

Seven days later, these animals were similarly treated with DPA in a final experiment in which, instead of BMI, morphine was used.

In a second group, rats (n=28) were lesioned (1 mA, 45 sec.) in the Ac and the Cm-Pf on the same coordinates as those given above, with a platinum electrode (anode), varnished except for the tip of 0.25 mm. The stereotaxic frame served as the cathode. In control animals, the electrode was driven to 1 mm above the coordinates used without applying current. The animals were

Table 1.

Behavioural effects of DPA-injections into different brain areas

Brain area		Coordinates AP	L	H	Behavioural effects Body shakes	Enhanced Activity	Chewing
Nucleus Accumbens	(2)	+9.8	1.2	-0.6	-	-	-
Nucleus Caudatus Anterior	(2)	+9.2	2.7	+0.4	-	-	-
Nucleus Caudatus Posterior	(2)	+7.5	3.5	+0.4	-	-	-
Centre Median	(12)	+4.0	0.8	-1.0	+	+	-
Substantia Nigra	(2)	+2.9	1.5	-2.2	-	-	-
Amygdala Centralis	(11)	+4.4	3.5	-2.4	-	-	+
Hippocampus	(4)	+2.8	3.0	+1.8	-	-	-
Locus Coeruleus	(4)	-0.8	1.0	-0.5	-	-	-

Numbers between brackets represent the number of animals.
Coordinates were derived from König and Klippel (1963).

treated with DPA (300 mg/kg i.p.) two weeks after lesioning and their behaviour was observed as described above.

At the end of each experiment rats were perfused with 10% formalin and either the position of the cannulae or the extent of the lesions was histologically verified (60 µm slices, cresylviolet stain).

Statistical comparison of the effects of the different treatments was carried out using the Mann-Whitney U-test. For the experiments involving intracerebral administration of BMI or morphine, the scores for body shakes, as well as those for horizontal activity, were combined for the period from the 7th to the 15th minute (or for fractions of this period), and then compared with control treatment over the same period.

Di-n-propylacetate (sodium valproate, Albic) was dissolved in artificial cerebrospinal fluid (CSF), according to Palaic et al. (1967), when intended for intracerebral injection. Intraperitoneal DPA was given as a 100 mg/ml solution in deionized water at a dose of 300 mg/kg. Bicuculline-methiodide (BMI, Pierce) and strychnine-nitrate were dissolved in deionized water and administered at the doses described under Results. Morphine.HCl (OPG) was dissolved in saline.

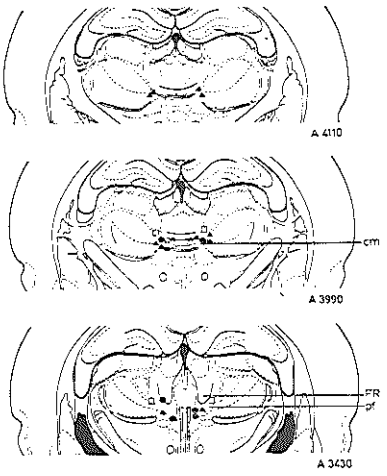

FIG. 1. Localization of the injection sites of DPA in the area of the centre median-nucleus parafascicularis (Cm-Pf). The symbols represent the efficiency of that particular site to induce body shakes: □, no body shakes; Δ, 1-4 body shakes; ●, > 4 body shakes. The figures are drawn from the Atlas of König and Klippel (1963) and the anterior level of each section is indicated. Abbreviations: cm, centre median; pf. nucleus parafascicularis; tvm, ventromedial nucleus; FR, fasciculus retroflexus.

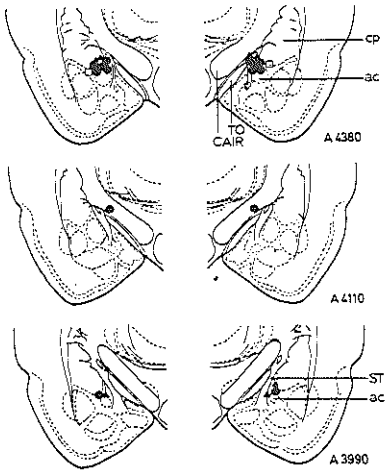

FIG. 2. Localization of the injection sites of DPA in the area of the central amygdala. The symbols represent the efficiency of that particular site to induce episodes of chewing: □, no chewing; Δ, 1-4 episodes of chewing; ●, > 4 episodes of chewing. The figures are drawn from the Atlas of König and Klippel (1963). The anterior level of each section is indicated. Abbreviations: ac, central amygdala nucleus; am, medial amygdala nucleus; ST, stria terminalis; cp, caudata putamen; TO, optical tract; CAIR, capsula interna reticulata.

Intracerebral injection of di-n-propylacetate

Intracerebral injection of DPA into eight different areas revealed that in only two areas, the Cm-Pf and the Ac, abstinence symptoms could be evoked, namely body shakes, enhanced locomotor activity and chewing (Table 1). The behavioural responses elicited from the Ac and Cm-Pf differed significantly (Table 2). The scores for swallowing, teeth chattering and head shakes did not differ significantly (data not shown).

The injection sites in the Cm-Pf are presented in Fig. 1 and the efficacy of each site for eliciting body shakes was plotted on frontal planes. Injection sites evoking a high number of body shakes were located in the centre median and in the lower part of the nucleus parafascicularis, somewhat posterior to the centre median.

Injection of DPA into Ac resulted in significantly more episodes of chewing when compared to the Cm-Pf area. These injection sites are shown in Fig. 2.

Table 2.

Effect on behaviour of intracerebral administration of DPA in the Ac and Cm-Pf

	Ac (11)	Cm-Pf (12)	
Horizontal activity	96 ± 10	148 ± 11	$p < 0.005$
Rearing	17,5 ± 3,1 (100)	41,7 ± 4,0 (100)	$p < 0.005$
Body shakes	0,8 ± 0,4 (36)	3,3 ± 0,9 (83)	$p < 0.05$
Chewing episodes	5,3 ± 1,3 (91)	0,9 ± 0,6 (42)	$p < 0.01$

Numbers between brackets at the head of the columns represent the number of animals. Numbers between brackets beside the scores represent the incidence in percentages. Mean values ± SEM are given.

Intracerebral administration of BMI after intraperitoneal DPA

DPA was administered intraperitoneally to rats and after 5 min BMI was given intracerebrally. BMI (0.5 µg), injected bilaterally into the Cm-Pf, suppressed body shakes by 92% ($p < 0.002$), whereas 0.2 µg gave about 65% suppression ($p < 0.05$) (Fig. 3). Injection of 0.5 µg BMI, bilaterally, immediately suppressed horizontal activity by 55% ($p < 0.02$), an effect which was sustained for the first three minutes, while during the last three minutes of the observation period an increase in horizontal activity was observed using 0.2 µg (190%; $p < 0.01$). Injection of strychnine, 1.0 µg bilaterally, into the Cm-Pf neither affected body shakes nor horizontal activity evoked by intraperitoneal administration of DPA.

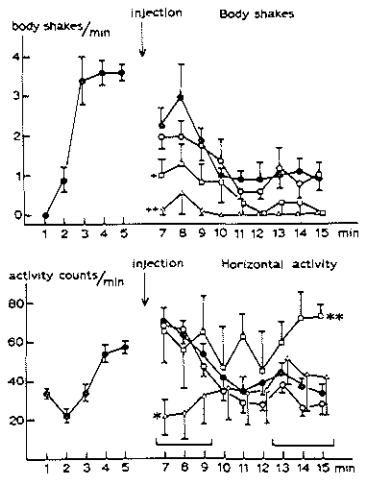

FIG. 3. Effects in rats of injection of BMI and strychnine into the Cm-Pf on body shakes and horizontal activity induced by DPA. At t = 0 DPA (300 mg/kg i.p.) was given and after 6 min BMI, strychnine or vehicle was administered intracerebrally. ●—●, control (n=9); □—□, BMI, 2 x 0.2 µg (n=4); Δ— Δ, BMI, 2 x 0.5 µg (n=5); o—o, strychnine, 2 x 1.0 µg (n=5). + $p < 0.05$; ++ $p < 0.002$; * $p < 0.02$ for the 7th to the 9th min; ** $p < 0.01$ for the 13th to the 15th min; all differences tested vs control. Bars represent SEM.

Intracerebral administration of BMI (0.5 µg bilaterally) into the Ac of DPA-pretreated rats resulted in a profound increase in body shakes (210%; $p < 0.005$). A lower dose (0.2 µg) was not effective. No significant changes in horizontal activity were observed (Fig. 4).

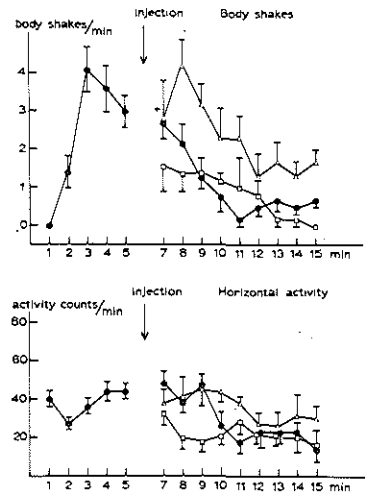

FIG. 4. Effects in rats of injection of of BMI into the Ac on body shakes and horizontal activity induced by DPA. At t = 0 DPA (300 mg/kg i.p.) was injected and after 6 min BMI or vehicle was administered intracerebrally. ●—●, control (n=6); □— □, BMI, 2 x 0.2 µg (n=5); Δ—Δ, BMI, 2 x 0.5 µg (n=6); + $p < 0.005$ vs control treatment. Bars represent SEM.

Intracerebral injection of morphine after intraperitoneal DPA

Rats received morphine intracerebrally 5 min after pretreatment with DPA (300 mg/kg i.p.). After bilateral injection of 0.5 µg morphine into the Cm-Pf, body shakes were suppressed by 72% ($p < 0.01$) and horizontal activity by 37% ($p < 0.002$) (Fig. 5). No suppression of body shakes or locomotor activity was observed after injection of morphine into the Ac, using doses of 0.5, 1.0 or 5.0 µg, bilaterally (Fig. 6). However, over the period from the 7th to the 11th min an increase in horizontal activity was observed using 0.5 µg (230%; $p < 0.01$) as well as 5.0 µg morphine (180%; $p < 0.05$).

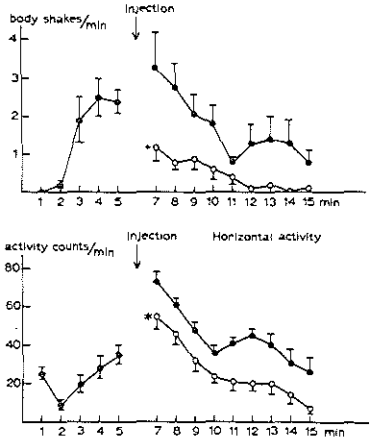

FIG. 5. Effects in rats of injection of morphine into the Cm-Pf on body shakes and horizontal activity induced by DPA. At t=0 DPA (300 mg/kg i.p.) was injected and after 6 min morphine or vehicle was administered intracerebrally. ●—●, control (n=7); o—o morphine, 2 x 0.5 µg (n=8). + $p < 0.01$; * $p < 0.002$ vs control. Bars represent SEM.

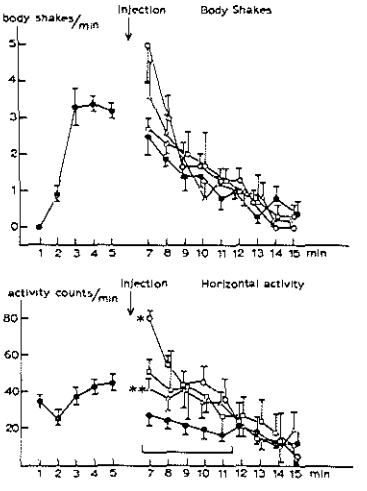

FIG. 6. Effects in rats of injection of morphine into the Ac on body shakes and horizontal activity induced by DPA. At t=0 DPA (300 mg/kg i.p.) was injected, and after 6 min morphine or vehicle was administered intracerebrally. ●—●, control (n=8); o—o, morphine, 2 x 0.5 µg (n=3); □—□, morphine, 2 x 1.0 µg (n=3); Δ—Δ, morphine, 2 x 5.0 µg (n=5). * $p < 0.01$; ** $p < 0.05$ vs control for the 7th to the 11th min. Bars represent SEM.

Bilateral lesions in the amygdala and thalamus

Destruction of the Ac significantly reduced ($p < 0.05$) the number of body shakes, when compared to sham-operated animals, i.e. 21.6 ± 1.8 (n=5) vs 34.2 ± 3.7 (n=9), respectively, after injection of DPA (300 mg/kg i.p.). Other symptoms were not affected. Lesions in the Cm-Pf did not have any effect on DPA-evoked behaviour. The extent of the lesions is shown in figures 7 and 8.

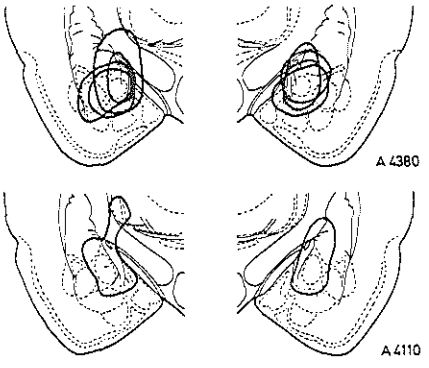

FIG. 7. Extent of damage induced by electrolytic lesions in the central amygdala. See the legend to Fig. 2 for abbreviations. The damage in each rat is shown bilaterally.

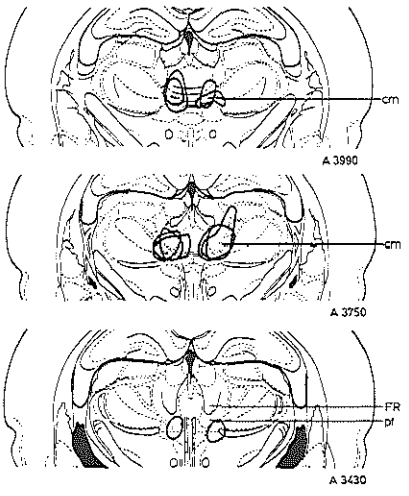

FIG. 8. Extent of damage induced by electrolytic lesions in the Cm-Pf. See the legend to Fig. 1 for abbreviations. The damage in each rat is shown bilaterally.

DISCUSSION

As has been shown previously, an intraperitoneal injection of DPA in rats results in quasi-morphine abstinence behaviour consisting mainly of centrally regulated symptoms (De Boer et al., 1977). These symptoms are probably evoked by increased GABA-ergic activity since they are prevented by GABA-antagonists (De Boer et al., 1977, 1980). A low dose of morphine was also found to suppress DPA-evoked behaviour.

The present data (Table 1), indicate that, of the eight brain structures studied, only the centre median-parafascicularis area (Cm-Pf) and the central amygdaloid nucleus (Ac) are involved in DPA-evoked abstinence behaviour. The possibility that GABA-ergic and opiate systems, present in the Cm-Pf and the Ac, form part of a regulating mechanism with regard to abstinence behaviour, seems, therefore, feasible.

GABA-ergic and opiate mechanisms in the Cm-Pf

A GABA-ergic system present in the Cm-Pf seems to be responsible, at least in part, for the induction of body shakes by intraperitoneal injection of DPA, since injection of DPA into this structure also evoked body shakes. Moreover, injection of the GABA-antagonist BMI, but not of the glycine antagonist strychnine into this area, suppressed the number of body shakes evoked after intraperitoneal injection of DPA.

The GABA-ergic system in the Cm-Pf, involved in the increase of horizontal activity evoked by DPA, differs from that involved in the body shakes, since, after injection of 0.2 µg BMI into the Cm-Pf, the duration of horizontal activity was extended, while the same dose suppressed the number of body shakes.

Stimulation of opiate receptors by injection of morphine into the Cm-Pf, suppressed the number of body shakes as well as the increase in horizontal activity evoked by intraperitoneally injected DPA. Although it has been suggested that morphine, in high doses, may act as a GABA-antagonist (Dingledine et al., 1977), it has been shown more recently that morphine may inhibit GABA-release, either by a presynaptic mechanism or by inhibition of the firing rate of GABA-ergic cells (Nicoll et al., 1980; Van der Heyden et al.,1980). This may explain the similar effects of BMI and morphine on DPA-evoked body shakes after their injection into the Cm-Pf. However, a primary involvement of GABA-ergic systems in the Cm-Pf in DPA-evoked behaviour can be excluded , as destruction of the Cm-Pf did not affect any of the symptoms.

Thus, GABA-ergic neurons present in the Cm-Pf seem to be facilitatory in the expression of DPA-induced body shakes, while their action can be counteracted by opiate mechanisms in the same area.

GABA-ergic and opiate mechanisms in the Ac

Although injection of DPA into the Ac resulted in enhanced chewing, no further evidence could be obtained for the mediation of this effect by GABA-ergic neurons, since BMI injected into the Ac had no effect on chewing evoked by intraperitoneal DPA-administration (data not shown).

Injection of BMI into the Ac increased the number of body shakes after intraperitoneal administration of DPA, suggesting the presence of an inhibitory GABA-ergic system in the Ac, which affects body shaking.

It has been reported that the Ac contains a high density of opiate receptors (Atweh and Kuhar, 1977b),probably connected to receiver cells of an enkephalinergic system (Gros et al., 1978; Simantov et al., 1977). Nevertheless, morphine injected into this brain area was without effect on DPA-evoked body shakes. Thus, the Ac contains GABA-ergic mechanisms which inhibit the expression of body shakes and which are unaffected by morphine.

The present results, therefore, suggest that, as far as body shakes are concerned, a GABA-ergic system in the Cm-Pf plays a facilitatory role, while a GABA-ergic system in the Ac exerts an inhibitory effect. Systemic injection of DPA may, thus, evoke abstinence behaviour by activating the GABA-ergic system in the Cm-Pf which may stimulate an unknown neuronal pathway responsible for the expression of body shakes, probably via disinhibition of this pathway. The GABA-ergic cells in the Cm-Pf might be under control of an opioid system.

The inhibitory action of the GABA-ergic system in the Ac on DPA-evoked behaviour and the fact that destruction of this structure decreases the number of body shakes, suggests that the Ac contains excitatory neurons which might be primarily involved in evoking body shakes. The GABA-ergic neurons present in this structure may inhibit these neurons directly.

The present data, obtained with rats in which quasi-morphine abstinence behaviour was induced by DPA, might have implications for the localization of the brain area responsible for the true morphine withdrawal syndrome. Several structures have been reported to be involved in the expression of morphine abstinence behaviour, e.g. areas located on the floor of the 4th ventricle (Laschka and Herz, 1977), the periaqueductal grey (Laschka and Herz, 1977; Wei et al., 1975) and the hypothalamus (Kerr and Pozuelo, 1971;

Wei et al., 1975). In none of these studies were the data related to a specific transmitter system. Amygdaloid structures have also been implicated in the morphine withdrawal syndrome (Calvino et al., 1979; Lagowska et al., 1978).

Furthermore, it has been reported that morphine alters the synthesis and distribution of GABA in the thalamus, although, after chronic administration of morphine, no differences were observed (Kuriyama and Yoneda, 1978; Yoneda et al., 1977). Further experiments are necessary to elucidate the role of GABA-ergic mechanisms in the Ac and the Cm-Pf in the morphine withdrawal syndrome.

In conclusion, the present study suggests that GABA-ergic systems, especially those in the Cm-Pf and the Ac, are involved in the expression of body shakes and horizontal activity evoked by DPA. An opioid input present in the Cm-Pf may regulate this system. It is possible that the projection of the Pf into the central amygdala, which has been described recently (Ottersen and Ben-Ari, 1979), is implicated in this system.

REFERENCES

Atweh, S.F., Kuhar, M.J.(1977a) Autoradiographic localization of opiate receptors in rat brain. II. The Brain Stem. Brain Res. 129, 1-12

Atweh, S.F., Kuhar, M.J.(1977b) Autoradiographic localization of opiate receptors in rat brain. III. The Telencephalon. Brain Res. 134, 393-405

Calvino, B., Lagowska, J., Ben-Ari, Y.(1979) Morphine withdrawal syndrome: differential participation of structures located within the amygdaloid complex and striatum of the rat. Brain Res. 177, 19-34

De Boer, Th., Bartels, K., Metselaar, H.J., Bruinvels, J. (1980) Di-n-propylacetate-induced abstinence behaviour as a possible correlate of increased GABA-ergic activity in the rat. Psychopharmacol. 71, 257-267

De Boer, Th., Metselaar, H.J., Bruinvels, J. (1977) Suppression of GABA-induced abstinence behaviour in naive rats by morphine and bicuculline. Life Sci. 20, 933-942

Dingledine, R., Iversen, L.L., Breuker, E. (1977) Naloxone as a GABA-antagonist: evidence from iontophoretic, receptor binding and convulsant studies. Eur. J. Pharmacol. 47, 19-27

Elchisak, M.A., Rosecrans, J.A. (1979) Development of morphine tolerance and physical dependence in rats depleted of brain catecholamines by 6-hydroxydopamine. Neuropharmacol. 18, 175-182

Godin, Y., Heiner, L., Mark, J., Mandel, P. (1969) Effects of di-n-propylacetate, an anticonvulsive compound, on GABA metabolism. J. Neurochem. 16, 869-873

Gros, C., Pradelles, P., Humbert, J., Dray, F., LeGal LaSalle, G., Ben-Ari, Y. (1978) Regional distribution of met-enkephalin within the amygdaloid complex and bed nucleus of the stria terminalis. Neuroscience Lett., 10, 193-196

Kerr, F.W.L., Pozuelo, J. (1971) Suppression of physical dependence and

induction of hypersensitivity to morphine by stereotaxic hypothalamic lesions in addicted rats. Mayo Clin. Proc. 46, 653-665
König, J.F.R., Klippel, R.A. (1963) The Rat Brain: A stereotaxic atlas of the forebrain and lower parts of the brain stem. The Williams and Wilkins Comp., Baltimore
Kuriyama, K., Yoneda, Y. (1978) Morphine-induced alterations of γ-amino-butyric acid and taurine contents and L-glutamate decarboxylase activity in the spinal cord and thalamus: possible correlates with analgesic action of morphine. Brain Res. 148, 163-179
Lagowska, J., Calvino, B., Ben-Ari, Y. (1978) Intra-amygdaloid applications of naloxone elicits severe withdrawal signs in morphine-dependent rats. Neuroscience Lett., 8, 241-245
Laschka, E., Herz, A. (1977) Sites of action of morphine involved in the development of physical dependence in rats.Psychopharmacol. 53, 33-37
Nicoll, R.A., Alger, B.E., Jahr, C.E. (1980) Enkephalin blocks inhibitory pathways in the vertebrate CNS. Nature (London), 287, 22-25
Ottersen, O.P., Ben-Ari, Y. (1979) Afferent connections to the amygdaloid complex of the rat and cat. I. Projections from the thalamus. J. Comp. Neurol. 187, 401-424
Palaic, D., Page, I.H., Khairallah, P.A. (1967) Uptake and metabolism of ^{14}C serotonin in rat brain. J. Neurochem. 14, 63-69
Pert, A. (1978) Central sites involved in opiate actions. In: J. Fishman (ed.) The bases of addiction, Dahlem Konferenzen, Berlin, pp. 299-322
Simantov, R., Kuhar, M.J., Uhl, G.R., Snyder, S.H. (1977) Opioid peptide enkephalin: Immunohistochemical mapping in rat central nervous system. Proc. Nat. Acad. Sci. U.S.A. (Wash.) 74, 2167-2171
Simler, S., Cieselski, L., Maitre, M., Randrianarisoa, H., Mandel, P. (1973) Effect of sodium n-dipropylacetate on audiogenic seizures and brain γ-aminobutyric acid level. Biochem. Pharmacol. 22, 1701-1708
Van der Heyden, J.A.M., Venema, K., Korf, J. (1980) *In vivo* release of endogenous GABA from rat striatum: effects of muscimol, oxotremorine and morphine. J. Neurochem. 34, 1648-1653
Van der Laan, J.W., De Boer, Th., Bruinvels, J. (1979) Di-n-propylacetate and GABA-degradation. Preferential inhibition of succinic semialdehyde dehydrogenase and indirect inhibition of GABA-transaminase. J. Neurochem. 32, 1769-1780
Van der Laan, J.W., Jacobs, A.W.C.M., Bruinvels, J. (1980) Effects of branched-chain fatty acids on GABA-degradation and behavior: Further evidence for a role of GABA in quasi-morphine abstinence behavior. Pharmacol. Biochem. Behav. 13, 843-849
Wei, E., Loh, H.H., Way, E.L. (1972) Neuroanatomical correlates of morphine dependence. Science, 177, 616-617
Wei, E., Loh, H.H., Way, E.L. (1973) Brain sites of precipitated abstinence in morphine-dependent rats. J. Pharmacol. Exp. Ther. 185, 108-115
Wei, E., Sigel, S.S.R., Loh, H.H., Way, E.L. (1975) Central sites of naloxone-precipitated shaking in the anaesthetized morphine-dependent rat. J. Pharmacol. Exp. Ther. 195, 480-487
Yoneda, Y., Kuriyama, K., Kurihara, E. (1977) Morphine alters distribution of GABA in thalamus. Brain Res. 124, 373-378

CHAPTER VI

DIPROPYLACETATE-INDUCED QUASI-MORPHINE ABSTINENCE BEHAVIOUR IN THE RAT: PARTICIPATION OF THE LOCUS COERULEUS SYSTEM

J.W. van der Laan, J. Bruinvels and A.R. Cools

Brain Research, submitted for publication

ABSTRACT

The role of the noradrenergic system in quasi-morphine abstinence behaviour induced by di-n-propylacetate (DPA) in rats has been studied. Depletion of noradrenaline (NA), by treatment with FLA-63, decreased the number of body shakes and the extent of horizontal activity evoked by DPA. Almost total suppression of these symptoms was obtained by injection of 20 ng morphine bilaterally into the locus coeruleus (LC). Destruction of the LC system by electrolytic lesion of the LC or by injection of 6-OHdopamine into the dorsal bundle revealed that degeneration of the NA system by at least 80% appears to be necessary to decrease the number of body shakes. It is concluded that the noradrenergic LC system fulfils a modulatory role in quasi-morphine abstinence behaviour induced by DPA.

INTRODUCTION

Systemic administration of di-n-propylacetate (DPA) induces a behavioural syndrome in rats consisting of body shakes, escape digging, hunchback posture, enhanced locomotor activity and various other symptoms (Bruinvels et al., 1980; De Boer et al., 1977, 1980).

Increased GABA-ergic activity is held responsible for the induction of this behaviour since it can be suppressed by the GABA-antagonists bicuculline and picrotoxin, but not by the glycine antagonist strychnine (De Boer et al., 1977, 1980). DPA is thought to enhance GABA-ergic activity by inhibition of succinic-semialdehyde dehydrogenase, the second enzyme in the degradation of GABA (Van der Laan et al., 1979).

Since the DPA-evoked behaviour resembles that seen during morphine abstinence (De Boer et al., 1977) it is referred to as quasi-morphine abstinence behaviour as defined by Collier (1974).

We have now used the DPA-induced behavioural syndrome as a model to study the role of the noradrenergic locus coeruleus (LC) system in morphine withdrawal behaviour. Participation of brain noradrenaline (NA) in morphine abstinence behaviour is suggested by several lines of evidence as reviewed recently (Iwamoto and Way, 1979). Contradictory results, however, have been an obstacle to the elucidation of the exact role of this transmitter (see Iwamoto and Way, 1979). These contradictions may, in part, be accounted for by differences in the species studied, in opiate treatment schedules, in scoring systems for the degree of abstinence behaviour and in the indicator of NA functionality. For instance, some investigators have studied steady state concentration of NA in parts of or in the whole brain, while others have studied turnover of NA by determination of its rate of synthesis or of degradation.

The role of the noradrenergic system in morphine abstinence behaviour seems to be important in view of recent reports on the use of clonidine in the detoxification of opiate addicts (Gold et al., 1978, 1979, 1980; Washton et al., 1980). Evidence has been presented that clonidine counteracts morphine abstinence behaviour by stimulation of α-adrenoceptors located in the LC (Aghajanian, 1978). Therefore, we decided to restrict ourselves, in the present study, mainly to the noradrenergic system originating in the LC, which projects via the dorsal bundle into the forebrain. To allow comparison with treatments that affect the whole brain NA content, a study on the effects of systemically administered FLA-63, a dopamine-β-hydroxylase inhibitor, was included.

In the present paper, the effect of changes in LC activity on DPA-induced behaviour has been examined using three different approaches: a) local injection of morphine into the LC; b) electrolytic lesion of the LC; c) chemical lesion of the LC forebrain projection.

MATERIALS AND METHODS

Sixty-eight male Wistar rats (150-225 g; TNO, Zeist, the Netherlands) were used for all experiments. In the first experiment rats (n=15) were treated with FLA-63 (20 mg/kg i.p.) three times at two hourly intervals. Two hours after the last injection they received DPA (300 mg/kg i.p.) and their behaviour was observed as follows. Rats were placed in a macrolon cage (47 x 27 x 15 cm) with sawdust bedding and were allowed to habituate for at least 30 min, whereafter DPA was administered intraperitoneally.

Behavioural observation was started directly after the injection and lasted for 15 min. The following signs were scored as absent, mild or marked: hunchback posture, piloerection, ptosis, penis licking and erection. Other signs were counted as quantal events: body shakes, head shakes, escape digging and foreleg shakes. Horizontal activity was measured simultaneously using an Animex activity meter.

A second group of animals (n=28) was anaesthetized with pentobarbital (60 mg/kg i.p.), placed in a stereotaxic frame and stainless steel cannulae were implanted bilaterally, as previously described by Pijnenburg et al. (1976). The following coordinates were chosen: AP -0.8, L 1.2, H 3.1, according to the coordinate system of König and Klippel (1963). The cannulae were inclined at an angle of 15^{o} from the posterior side and 5^{o} from the lateral side to avoid bleeding. After a recovery period of one week, during which the animals were housed individually, behavioural observations were carried out after placing the rats in a wooden cage (50 x 30 x 30 cm) with a single transparent perspex wall, on sawdust bedding. Behavioural scoring after injection of DPA (300 mg/kg i.p.) was carried out as described above, except for the horizontal activity, which was scored as the number of full cage lenghts (50 cm) the rats had covered. After five minutes morphine or saline was injected intracerebrally in a volume of 0.5 µl, the latter serving as a control. To check the correct placement of the cannulae, histological examination was carried out as described previously (Van der Laan and Bruinvels, in press).

In a third experiment, rats (n=12) were anaesthetized with chloral hydrate (400 mg/kg i.p.), placed in a stereotaxic frame and brain lesions were performed centered on the LC, AP -0.5, L 0.9, H 2.6, according to the coordinate system of König and Klippel (1963), with a platinum electrode (anode) varnished except for the tip of 0.25 mm. The stereotaxic frame served as the cathode. The electrode was inclined at an angle of 16^{o} from the posterior side. The intensity of the current was 1 mA and the duration 45 sec. In control animals the electrode was driven to 2 mm above the LC without applying current. Two weeks after lesioning, the animals were treated with DPA (300 mg/kg) and their behaviour was observed for 15 min, as described above. In this experiment, horizontal activity was measured using a Varimex activity meter.

In a fourth group of rats (n=13), under chloral hydrate anaesthesia, 6-OHdopamine was injected bilaterally into the dorsal bundle, AP 1.8, L 1.0, H 0.5 (König and Klippel, 1963). 6-OHdopamine was administered in a

dose of 8 µg in 2 µl, bilaterally, and was dissolved in deionized water containing 0.2 mg/ml ascorbic acid. Controls received only the ascorbic acid solution. The animals were housed five per cage and were allowed to recover for 21 days. Subsequently, the rats were treated with DPA, whereafter their behaviour was observed for 15 min, as described above.

The lesioned rats and the 6-OHdopamine treated rats were decapitated on the fourth day after the injection of DPA. Brains were rapidly removed and dissected as follows. A transverse section was made just anterior to the optic chiasm keeping the upper surface of the brain horizontal. The total anterior part was used for analysis in the lesioned rats. In the rats treated with 6-OHdopamine, the cortex was dissected free from this forebrain according to Gispen et al. (1972). The cortex tissue was frozen on dry ice and noradrenaline was determined according to Westerink and Korf (1977).

Statistical comparison between different treatments was carried out using the Mann-Whitney U-test (Siegel, 1956). For the experiments with intracerebral administration of morphine, the sum of the scores for body shakes or horizontal activity over the total period from the 7th to the 15th min were compared with control values obtained over the same period.

DPA (sodium valproate, Depakine^{R}) was purchased from Albic BV, Maassluis, the Netherlands, and dissolved in deionized water to a concentration of 100 mg/ml. 6-OHdopamine and FLA-63 were obtained from Labkemi AB, Sweden. FLA-63 (bis-(1-methyl-4-homopiperazinyl-thiocarbonyl)disulfide) was dissolved in 2N HCl, diluted with saline, the pH then adjusted to 6 with 2N NaOH, and injected at a concentration of 2.5% w/v.

RESULTS

Intraperitoneal injection of DPA mainly resulted in the appearance of body shakes and enhanced locomotor activity, while hunchback posture and piloerection were also observed in nearly all animals. Other symptoms occurred at a much lower incidence and were, in most cases, not considered any further.

Pretreatment of the animals with FLA-63 prevented the full expression of DPA-induced behaviour (Table 1). Both the number of body shakes and the horizontal activity were decreased significantly by about 58 and 38%, respectively. Escape digging was totally suppressed. Other symptoms were not altered significantly.

Table 1.

Effects of pretreatment with FLA-63 on the behaviour evoked by DPA

	Control (8)	FLA-63 (7)	% inhibition
Body shakes	16.6 ± 2.7	7.0 ± 2.0*	58
Escape digging	6.9 ± 1.6	0.3 ± 0.1*	96
Horizontal activity	697 ± 75	434 ± 67*	38

Mean values ± SEM are given. Numbers between brackets represent the number of animals tested. Statistical significance (* $p < 0.02$) was calculated using the Mann-Whitney U-test (two tailed) (Siegel, 1956).

Injection of 20 ng morphine (bilaterally) into the LC, 5 min after injection of DPA, resulted in a profound decrease in body shakes and horizontal activity (90%; $p < 0.001$), while after injection of 10 ng morphine only body shakes were suppressed by 30% ($p < 0.05$) (Fig. 1). Histological confirmation of the placement of the cannulae is given in Fig. 2. At least at one side and in most cases at both sides a good placement of the cannulae was achieved in all animals. No differences in location of the cannulae were observed between the groups receiving morphine or saline.

Electrolytic lesioning of the LC or injection of 6-OHdopamine into the dorsal bundle both caused a degeneration of the noradrenergic projection to the forebrain regions of the rats (Table 2). The treated animals were divided into several groups according to the remaining level of NA. Only in those rats with a forebrain NA concentration 20% of that of controls could a decrease of about 50% in the number of body shakes be observed.

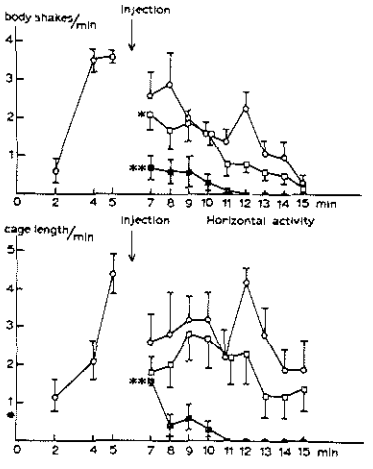

FIG. 1. Effects of injection of morphine into the locus coeruleus of the rat on body shakes and horizontal activity induced by DPA (300 mg/kg i.p.). At t=0 DPA was given and after 5 min morphine. HCl in two doses. o—o, control (n=9); □—□, morphine 10 ng, bilaterally (n=12); ■—■, morphine 20 ng, bilaterally (n=7); * $p < 0.05$; ** $p < 0.001$ vs control.

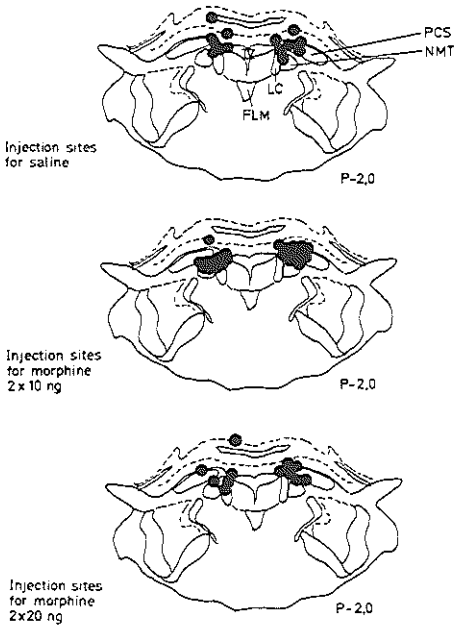

FIG. 2. Localization of the injection sites of morphine or saline in the area of the locus coeruleus. The two sites for each rat are given. The figures are drawn from the atlas of Pellegrino and Cushman (1971). The posterior level is 2.0 mm posterior to the plane through the interaural line.
Abbreviations: LC = locus coeruleus; PCS = pedunculus cerebellar superior; NMT = nucleus tractus mesencephalic nervi trigemini; FLM = fasciculus longitudinalis medialis.

A much smaller, not significant, decline in horizontal activity was present (20%). In those rats with a higher residual brain NA concentration neither a decrease in body shakes nor in horizontal activity could be detected.

Table 2.

Relation between noradrenaline (NA) concentration in the LC projection to the forebrain and DPA-induced body shakes and horizontal activity

A Electrolytic lesion of the LC

	NA concentration in forebrain in %	Body shakes in %	Horizontal Activity in %
Sham (5)	100 ± 8	100 ± 8	100 ± 8
50 < NA < 90% (5)	64 ± 5	82 ± 10	102 ± 6
NA < 25% (2)	18 (14, 21)	53 (39, 67)	81 (59, 103)

B Chemical lesion 6-OHDA of the dorsal bundle

	NA concentration in cortex in %	Body shakes in %	Horizontal Activity in %
Sham (5)	100 ± 4	100 ± 13	100 ± 15
NA ≈ 100% (3)	95 ± 3	118 ± 19	95 ± 13
25 < NA < 50% (3)	42 ± 5	82 ± 11	84 ± 20
NA < 25% (2)	19 (18, 20)	47 (45, 49)	76 (80, 71)

Values given represent the percentages of the appropriate control values and are given as means ± SEM.
A: results obtained with rats which received an electrolytic lesion in the LC.
B: results obtained with rats which received an injection of 6-OH dopamine into the dorsal bundle.
Absolute values for the controls: NA concentration A (forebrain) 121 ± 9.9 ng/g; B (cortex) 158.0 ± 5.8 ng/g; Body shakes: A 35.8 ± 2.7; B 26.8 ± 3.6; Horizontal activity: A 260.0 ± 19.6; B 119.5 ± 17.4.
Numbers between brackets represent the number of animals.

DISCUSSION

The present results suggest that the LC is intimately involved in the expression of quasi-morphine abstinence behaviour induced by DPA.

According to the data described by Herz et al. (1974) the treatment schedule used in the experiment with FLA-63 would be expected to deplete the NA level in brain to about 25%. The results obtained in this experiment, therefore, indicate that an intact noradrenergic system is required for the expression of DPA-induced abstinence behaviour as has been shown for true morphine abstinence behaviour (Herz et al., 1974).

Since the noradrenergic pathway originating from the LC is one of the main noradrenergic systems in the brain, we have tried to modulate noradrenergic activity by injecting morphine directly into this nucleus. Systemic

administration of morphine has been previously shown to suppress DPA-evoked behaviour (De Boer et al., 1977, 1980).

In addition, morphine administered either intravenously or microiontophoretically into the LC, has been shown to inhibit LC neuronal activity (Aghajanian, 1978; Bird and Kuhar, 1977; Korf et al., 1974). Therefore, injecting morphine into this area might be expected to affect DPA-evoked behaviour. The results obtained indeed showed that an active noradrenergic LC system, projecting into the forebrain, is a prerequisite for the full expression of DPA-evoked body shakes, since inhibition of the LC activity by morphine as well as an 80% degeneration of the LC forebrain projection resulted in a profound decrease in the incidence of this symptom (Fig. 1, Table 2). These results, therefore, suggest that impairment of the activity of dorsal noradrenergic bundle can be held responsible for the suppression of body shakes by FLA-63.

The high sensitivity of the LC to the body shakes-suppressing effect of morphine becomes apparent when compared to the doses required to give the same effects following injection into the centre median-parafascicularis area (Cm-Pf) and the central nucleus of the amygdala (Ac) (Van der Laan and Bruinvels, in press). When morphine was injected into the Cm-Pf a 70% suppression of body shakes was obtained using a dose of 0.5 µg (bilaterally), whereas no suppressive effect of morphine (at doses up to 5.0 µg, bilaterally) could be found following injection into the Ac. The relative sensitivity of these structures for the suppression of body shakes by morphine agrees very well with that reported by Wei et al. (1975), who studied the effect of morphine on wet shake behaviour of anaesthetized rats immersed into cold water.

The observation that a decrease of at least 80% in the NA level in the forebrain or cortex appears to be necessary to affect the quasi-morphine abstinence behaviour induced by DPA, is in agreement with results obtained by Herz et al. (1974), who studied levallorphan precipitated morphine withdrawal in rats. These authors, using α-methyl, p-tyrosine methyl ester and FLA-63 to deplete NA levels, reported a strong suppression of body shakes only when brain NA levels were decreased more than 70%.

Since injection of DPA itself (20 µg, bilaterally) into the LC does not evoke any of the behavioural symptoms as can be observed after intraperitoneal injection (Van der Laan and Bruinvels, in press), it seems unlikely that a GABA-ergic mechanism in this region is directly involved in the induction of this syndrome. Recent studies, however,

that GABA-ergic systems located in the central amygdala and the Cm-Pf may be primary involved in DPA-evoked behaviour (Van der Laan and Bruinvels, in press). Nevertheless, the present data indicate the requirement of an active LC system in this behaviour. Such a condition is compatible with the findings of Aghajanian (1978) demonstrating an increased LC firing rate during morphine withdrawal in rats, which could be suppressed by clonidine. Treatment with clonidine, which has been reported to suppress abstinence behaviour in rats and man (Fielding et al., 1978; Gold et al., 1980; Vetulani and Bednarczyk, 1977; Washton et al., 1980) also reduced NA turnover in the cerebral cortex of morphine dependent rats treated with naloxone (Crawley et al., 1979; Laverty and Roth, 1980).. Therefore, the evidence presented suggests that DPA-evoked behaviour not only resembles morphine abstinence behaviour in rats, but also seems to be mediated by an identical mechanism, in sofar as the noradrenergic dorsal bundle from the LC is involved.

The requirement of NA for DPA-evoked behaviour can be understood in the light of recent reports on noradrenergic potentiation of GABA-ergic inhibition, both in the somatosensory cortex and the cerebellum of rats, without effect on spontaneous discharge (Moises and Woodward, 1980; Waterhouse et al., 1978; Woodward et al., 1979). The authors suggested that NA may act as a neuromodulator, enhancing the efficiency of a cell to recognize important signals amidst background noise, rather than as a classical neurotransmitter. Such a role for NA in the hippocampus has also been described (Segal and Bloom, 1976). It is possible, therefore, that total suppression of LC activity by injection of morphine into this area prevents essential noradrenergic potentiation of DPA-enhanced GABA-ergic activity.

REFERENCES

Aghajanian,G.K. (1978) Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. Nature, 276, 186-188

Bird, S.J., Kuhar, M.J. (1977) Iontophoretic application of opiates to the locus coeruleus. Brain Res. 122, 523-533

Bruinvels, J., Van der Laan, J.W., De Boer, Th. (1980) GABA degradation and its possible relationship to the "Morphine Abstinence" syndrome. In: H. Lal (ed.) GABA Neurotransmission, Brain Res. Bull. Vol. 5, suppl. 2, pp. 797-804

Collier, H.O.J. (1974) The concept of the quasi-abstinence effect and its use in the investigation of dependence mechanisms. Pharmacology 11, 58-61

Crawley, J.N., Laverty, R., Roth, R.H. (1979) Clonidine reversal of increased norepinephrine metabolite levels during morphine withdrawal. Eur. J. Pharmac. 57, 247-250

De Boer, Th., Bartels, K., Metselaar, H.J., Bruinvels, J. (1980) Di-n-propylacetate-induced abstinence behaviour as a possible correlate of increased GABA-ergic activity in the rat. Psychopharmacol. 71, 257-267

De Boer, Th., Metselaar, H.J., Bruinvels, J. (1977) Suppression of GABA_ induced abstinence behaviour in naive rats by morphine and bicuculline. Life Sci. 20, 933-942

Fielding, S., Wilker, J., Hynes, M., Szewczak, M., Novick Jr., W.J., Lal, H. (1978) A comparison of clonidine with morphine for antinociceptive and antiwithdrawal actions. J. Pharmacol. Exp. Ther. 207, 899-905

Gispen, W.H., Schotman, P., De Kloet, E.R. (1972) Brain RNA and hypophysectomie; a topographical study. Neuroendocrinology 9, 285-296

Gold, M.S., Redmond Jr., D.E., Kleber, H.D. (1978) Clonidine blocks acute opiate withdrawal symptoms. The Lancet II, 599-602

Gold, M.S., Redmond Jr., D.E., Kleber, H.D. (1979) Noradrenergic hyperactivity in opiate withdrawal supported by clonidine reversal of opiate withdrawal. Am. J. Psychiatry 136, 100-102

Gold, M.S., Pottash, A.L.C., Extein, I., Kleber, H.D. (1980) Clonidine and opiate withdrawal. The Lancet II 1078-1079

Herz, A., Bläsig, J., Papeschi, R. (1974) Role of catecholaminergic mechanisms in the expression of the morphine abstinence syndrome in rats. Psychopharmacol. 39, 121-143

Iwamoto, E.T., Way, E.L. (1979) Opiate actions and catecholamines. In:Loh, H.H. Ross, D.H. (eds.) Neurochemical mechanisms of opiates and endorphins, Advances in Biochem.Psychopharmac.Vol. 20 Raven Press,New York,pp.357-407

König, J.F.R., Klippel, R.A. (1963) The Rat Brain: A stereotaxic atlas of the forebrain and lower parts of the brain stem. The Williams and Wilkins Comp. Baltimore

Korf, J., Bunney, B.S., Aghajanian, G.K. (1974) Noradrenergic neurons: morphine inhibition of spontaneous activity. Eur. J. Pharmac. 25, 165-169

Laverty, R., Roth, R.H. (1980) Clonidine reverses the increased norepinephrine turnover during morphine withdrawal in rats. Brain Res. 182, 482-485

Moises, H.C., Woodward, D.J. (1980) Potentiation of GABA inhibitory action in cerebellum by locus coeruleus stimulation. Brain Res. 182, 327-344

Pellegrino, L.J., Cushman, A.J. (1971) A Stereotaxic Atlas of the rat brain, Meredith, New York

Pijnenburg, A.J.J., Honig, W.M.M., Struyker Boudier, H.A.J., Cools, A.R., Van der Heyden, J.A.M., Van Rossum, J.M. (1976) Further investigations on the effect of ergometrine and other ergot derivatives following injection into the nucleus accumbens of rats. Arch. Int. Pharmacodyn. 222, 103-115

Segal, M., Bloom, F.E. (1976) The action of norepinephrine in the hippocampus IV. The effects of locus coeruleus stimulation on evoked hippocampal unit activity. Brain Res. 107, 513-525

Siegel, S. (1956) Nonparametric statistics for the behavioral sciences. McGraw-Hill Kogakusha, Tokyo

Van der Laan, J.W., Bruinvels, J. Dipropylacetate-induced quasi-morphine abstinence behaviour in the rat: Involvement of amygdaloid and thalamic structures. Brain Res. in press.

Van der Laan, J.W., De Boer, Th., Bruinvels, J. (1979) Di-n-propylacetate and GABA degradation. Preferential inhibition of succinic semialdehyde

dehydrogenase and indirect inhibition of GABA-transaminase. J. Neurochem. 32, 1769-1780
Vetulani, J., Bednarczyk, B. (1977) Depression of shaking behaviour elicited by nalorphine in morphine dependent rats. J. Pharm. Pharmac. 29, 567-569
Washton, A.M., Resnick, R.B., Rawson, R.A. (1980) Clonidine for outpatient opiate detoxification. The Lancet I 1078-1079
Waterhouse, B.D., Moises, H.C., Woodward, D.J. (1978) Noradrenergic modulation of somatosensory cortical neuronal responses to iontophoretically applied putative neurotransmitters. Soc. Neurosci. Abst. 4, 286
Wei, E., Sigel, S., Way, E.L. (1975) Regional sensitivity of the rat brain to the inhibitory effects of morphine on wet shake behavior. J. Pharmacol. Exp. Ther. 193, 56-63
Westerink, B.H.C., Korf, J. (1977) Rapid concurrent automated fluorimetric assay of noradrenaline, dopamine, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 3-methoxytyramine in milligram amounts of nervous tissue after isolation on Sephadex G-10. J. Neurochem. 29, 697-706
Woodward, D.J., Moises, H.C., Waterhouse, B.D., Hoffer, B.J., Freedman, R. (1979) Modulatory actions of norepinephrine in the central nervous system. Fed. Proc. 38, 2109-2116

CHAPTER VII

DIPROPYLACETATE-INDUCED QUASI-MORPHINE ABSTINENCE BEHAVIOUR: MECHANISMS AND SITE OF INDUCTION

GENERAL DISCUSSION

Mechanism of action of DPA

Whole brain GABA is increased in rats after DPA administration and the time course of this change matches that of the anticonvulsant effect of DPA (see section 1.2.6). Mandel and co-workers have reported that DPA inhibits GABA-T (Godin et al., 1969; Simler et al., 1973) and they have assumed that DPA exerts its GABA-enhancing effect through inhibition of this enzyme. However, the K_i of DPA for this enzyme was found to be rather high, 18 mM, a value of the same order as that found in our experiments (23.2 mM; chapter II). The present results indicate that DPA more potently inhibits SSA-DH (K_i= 0.5 mM), in agreement with the data reported by other authors (Harvey et al., 1975; Sawaya et al., 1975; Anlezark et al., 1976). Calculation of the kinetic constants for GABA-T led to the conclusion that the equilibrium of the reactions catalyzed by this enzyme is strongly biased towards GABA, confirming the data of Duffy et al. (1972), and that product inhibition by SSA is to be expected.

Therefore, the following mechanisms of action can be put forward. Administration of DPA will result in inhibition of SSA-DH, causing an accumulation of its substrate SSA. The accumulated SSA will prevent further degradation of GABA by inhibition of the GABA-T forward reaction or initiation of the GABA-T reversed reaction, thereby enhancing the GABA concentration in brain.

Previous reports had shown that the brain and plasma levels of DPA, which lead to a rise in GABA levels in the rat brain, are of the order of 0.1 - 1.0 mM (Sawaya et al., 1975). Since DPA has a much weaker inhibitory effect on GABA-T, it can now be clearly understood in which way DPA exerts its GABA-increasing action. The action of DPA on SSA-DH (K_i=0.5 mM) and the effects of the resulting accumulation of SSA explain the increase in GABA levels in a more satisfactory way. The question now arises as to whether this mechanism of action is also involved in DPA-evoked quasi-morphine abstinence behaviour.

Mechanisms involved in quasi-morphine abstinence behaviour

There is considerable evidence that DPA induces quasi-morphine abstinence behaviour via stimulation of GABA-ergic activity, since the behaviour can be suppressed by the GABA antagonists bicuculline and picrotoxin (see section 1.4.2). Therefore, we have studied the possibility that the mechanism of action of DPA, stated in the preceding section, is responsible for the induction of this behaviour. We have compared the biochemical and behavioural effects of analogues of DPA, namely 2,2-dimethylvaleric acid (DMV), 2-methyl,2-ethylcaproic acid (MEC) and caproic acid. It appeared that the two branched-chain fatty acids, but not caproic acid, are capable of inducing a similar behaviour pattern to that found with DPA. A correlation was found between the K_i's of these compounds for SSA-DH and the doses exerting a maximum effect on behaviour. However, MEC and DMV could not evoke the behaviour to the same extent as DPA, probably due to their concurrent inhibition of GABA-T. The maximum scores for body shakes and horizontal activity were found to be related to the ratio of the K_i's of these compounds for GABA-T over SSA-DH. Thus, concomitant inhibition of GABA-T by these compounds suppressed the behaviour, which was induced by inhibition of SSA-DH.

These results fit the hypothetical model, proposed by De Boer (1977), concerning the compartmentalized action of DPA, in inducing quasi-abstinence behaviour (see section 1.4.2; Fig. 1.4.2). According to this model, DPA increases GABA only in nerve endings, while the GABA-T inhibitor, amino-oxyacetic acid (AOAA), is mainly active in glial cells. Supporting evidence for this hypothesis has been obtained recently by Sarhan and Seiler (1979), who measured the GABA concentration in different subcellular organelles of mouse brain after administration of DPA. The data suggest that the DPA-induced increase in GABA is exclusively localized in the synaptosomal fraction, leaving non-synaptosomal GABA unchanged. In addition, Iadarola and Gale (1979) compared the effects of AOAA and DPA on GABA content in the substantia nigra of rats in which the striatonigral GABA-ergic projections had been unilaterally destroyed and provided evidence that the GABA increase produced by DPA is associated mainly with GABA-ergic nerve terminals.

The present data can be explained in a similar way, supposing a different action of the fatty acids in two distinct metabolic compartments of the brain. The SSA-DH located in nerve endings (see Fig. 1) may be more susceptible to inhibition by the fatty acids in comparison to its counterpart

in the glial cell and neuronal cell body compartment. The reverse seems to be true for the GABA-T in both compartments, the enzyme located in glial cells being more sensitive to the branched-chain fatty acids.

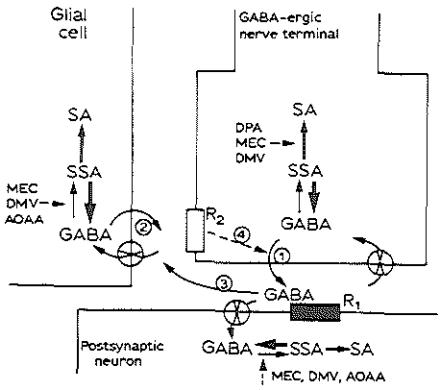

FIG.1. Hypothetical model about a GABA-ergic nerve terminal together with its surrounding cells. 1 = the release of GABA from the nerve terminal; 2 = release of GABA from a glial cell; 3 = diffusion of GABA to presynaptic areas; 4 = inhibitory effect of stimulation of a presynaptic autoreceptor (R_2) on the release of GABA by depolarization of the membrane; R_1 = post-synaptic GABA-receptor.

These different kinetic properties of the two forms of GABA-T in these two compartments of the brain may be explained by the existence of isozymes (Salganicoff and De Robertis, 1965; Ngo and Tunnicliff, 1978). The GABA accumulated in this way in the glial cell may be released from or leak out of these cells (Minchin and Iversen, 1974), subsequently stimulating presynaptic autoreceptors. Activation of these GABA-ergic autoreceptors will inhibit the further release of GABA (Mitchell and Martin, 1978; Van der Heyden et al., 1979; Brennan et al., 1981; see section 1.2.3).

This hypothesis also enabled us to explain the discrepancy between the time courses of the DPA-induced excitatory behaviour and the DPA-induced increase in GABA in brain (chapter IV). A second injection of DPA given shortly after the first was unable to evoke the abstinence to its full extent. Since the time course of this acute tolerance parallelled the increase in the GABA concentration in the brain, resulting from the first injection of DPA (see Fig. 1.2.6), a dual role for GABA was suggested in both initiation and termination of the behaviour. According to the model described above, DPA should act preferentially in the nerve terminal compartment, thus initiating abstinence behaviour by enhancement of GABA-

ergic transmission. On the other hand, the overflow of GABA to presynaptic areas (arrow 3 in Fig. 1) may activate presynaptic autoreceptors, thus terminating the abstinence behaviour through inhibition of the enhanced release of GABA. Recently, Sarhan and Seiler (1979) presented evidence for an overflow of GABA, indicating an increase in synaptosomal GABA during the first 10 min and thereafter, an increase in non-synaptosomal material. This time course for the increase in synaptosomal GABA after DPA is in agreement with the time course of the quasi-abstinence behaviour induced by DPA.

The finding that pretreatment with the dopamine-β-hydroxylase inhibitor FLA-63 prevented the full expression of the DPA-induced behaviour suggests that the presence of a normal level of noradrenaline (NA) is required for the induction of this behaviour (chapter VI). Since pretreatment of morphine-dependent rats with FLA-63 prevents also induction of body shakes by levallorphan, these data support the specificity of the DPA-induced behaviour as a model for the true abstinence behaviour and indicate that identical mechanisms may be involved. In recent years it has been established that NA may act as a neuromodulator enhancing the signal efficiency of GABA and probably also that of other neurotransmitters (Woodward et al., 1979; Moises and Woodward, 1980). As discussed in the next section, the noradrenergic bundle originating in the locus coeruleus appears to be particularly involved in the DPA-induced behaviour.

The data discussed so far, which indicate an important role of GABA-ergic activity in DPA-evoked abstinence behaviour, give rise to the question as to which mechanisms are involved in true morphine withdrawal. With respect to the involvement of the nerve terminal GABA compartment, two findings are important, namely the presynaptic inhibition of GABA-release by morphine (Van der Heyden et al., 1980; Corrigall and Linsemann, 1980) and the decreased activity of GAD after development of physical dependence (Tzeng and Ho, 1978; see section 1.3.4). Long-term inhibition of GABA-release by morphine may result in the development of supersensitivity of postsynaptic GABA-receptors (see section 1.2.3), as has been described recently (Ticku and Huffman, 1980). It may be suggested that after withdrawal of morphine an enhanced stimulation of these receptors will occur.

As far as GAD is concerned, the decreased activity found during the development of physical dependence is restored after withdrawal of morphine or precipitation of abstinence by naloxone, thus resulting in a relatively enhanced GAD activity when compared with the preceding phase (Tzeng and Ho,

1978). This relatively enhanced activity of GAD implies an enhanced GABA-ergic activity, which, combined with the supersensitivity of GABA-receptors, offers a GABA-ergic mechanism which may initiate abstinence behaviour.

No indications were found that a decrease in SSA-DH activity, the mechanism involved in the induction of quasi-abstinence by DPA, is also implicated in the expression in true morphine withdrawal behaviour. Repeated administration of increasingly high doses of morphine (50-200 mg/kg daily, during 14 days) did not result in a decreased SSA-DH activity or affinity for SSA, measured 48 h after the last injection of morphine (Van der Laan, Jacobs and Bruinvels, unpublished). However, it is not unlikely that a possible decreased activity of SSA-DH occurring only in the neuronal compartment, will be masked by the bulk of unchanged SSA-DH in the remaining cells.

We have tried to show that the present results concerning DPA-induced behaviour provided further insight into the mechanism which may be involved in true morphine abstinence behaviour.

Considering the present data in relation to those obtained using other quasi-abstinence models, e.g. phosphodiesterase inhibitors, TRH or EGTA (see section 1.3.2), it can be concluded that they are complementary, yielding insight into the complex mechanisms in morphine abstinence behaviour. The study on DPA and TRH may provide data concerning the involvement of intercellular mechanisms, on the level of neurotransmission or neuro-modulation, whereas the behaviour induced by phosphodiesterase inhibitors and EGTA may throw some light on the involvement of intracellular mechanisms in morphine abstinence behaviour.

Brain areas involved in quasi-morphine abstinence behaviour

Within the scope of this thesis, the involvement of several brain structures in the expression of DPA-induced quasi-morphine abstinence behaviour was investigated. Only three of them, namely the central amygdala (Ac), the centre median-parafascicularis area (Cm-Pf) and the locus coeruleus (LC) appeared to participate, each in a different way. The other areas, where DPA was without effect, were nucleus caudatus, nucleus accumbens, hippocampus and substantia nigra (chapters V and VI).

A GABA-ergic system in the Cm-Pf appears to have a facilitatory influence on body shakes and locomotor activity, since injection of DPA into this brain structure resulted in a large number of body shakes and greater

locomotor activity when compared with other areas. Administration of bicuculline methiodide (BMI) into the Cm-Pf suppressed body shakes evoked by intraperitoneal DPA, but had only minor effects on horizontal activity, whereas injection of morphine into the same structure suppressed both behavioural symptoms. A high amount of GAD appears to be present in the nucleus parafascicularis (Massari et al., 1976), which is activated after acute administration of morphine (Kuriyama and Yonenda, 1978). After chronic administration of morphine,tolerance to this effect occurs. Whether an enhanced or a decreased synthesis of GABA becomes apparent, remains to be investigated.

Furthermore, it may be suggested that an inhibitory influence on body shakes is provided in a GABA-ergic system in the Ac, since injection of BMI into the Ac shortly after intraperitoneal injection of DPA resulted in an increase in the number of body shakes. Morphine injected into this structure potentiated locomotor activity slightly, but had no effect on the body shakes evoked by DPA.

The Ac contains cell bodies of a GABA-ergic pathway projecting to the bed nucleus of the stria terminalis (BST). In addition, a recurrent GABA-ergic pathway exists with cell bodies in the BST and terminals in the Ac (Le Gal La Salle et al., 1978). Body shakes can also be induced by injection of kainic acid, a rigid analogue of glutamate, into this area (Ben-Ari et al.,1978; see Table 1.3.2).It can, therefore, be suggested that the neurons in the Ac, responsible for the induction of body shakes, can be excited physiologically by glutamate, and are under the inhibitory control of GABA (possibly originating in the BST). It is remarkable that DPA, while also increasing the level of GABA in the amygdala, can induce body shakes. It is not inconceivable that the GABA-projection from the BST to the Ac is, in turn, under GABA-ergic control in the BST (Ben-Ari et al., 1976). Administration of DPA to a rat, then, will cause an enhanced inhibition of the activity of the GABA-ergic pathway to the Ac, resulting in disinhibition of the neurons in the Ac. Subsequent injection of BMI into the Ac will cause a further disinhibition leading to a higher number of body shakes.

Thus, GABA-ergic systems in the Cm-Pf and in the Ac may have opposite roles in the initiation of DPA-induced quasi-morphine abstinence symptoms.

Since injection of DPA into the LC did not evoke quasi-morphine abstinence symptoms, the GABA-ergic system present in the LC (Guyenet and Aghajanian, 1978) does not appear to be directly involved in the induction of this syndrome. Nevertheless, the present data indicate that an active

LC system is a prerequisite for this behaviour. In the preceding section it was stated that depletion of NA with FLA-63 decreased the full expression of DPA-evoked behaviour. Therefore, impairment of the activity of the dorsal noradrenergic bundle may be held responsible for the suppression of body shakes by FLA-63. Injection of morphine into the LC, thus decreasing the LC firing rate (Bird and Kuhar, 1977) also suppressed DPA-induced behaviour almost totally. These data are in agreement with the findings of Aghajanian (1978), demonstrating an increased LC firing rate during morphine withdrawal in rats, which could be suppressed by clonidine. Clonidine has been reported to suppress abstinence behaviour in rats too (Fielding et al., 1978).

It has been demonstrated that the majority of the noradrenergic varicosities do not form synaptic contacts, but are located in gaps between glial and nerve cells, and the released NA probably affects a large number of neurons in the vicinity (Descarries et al., 1977). Furthermore, NA seems to act more as a neuromodulator than as a transmitter in the classical sense, i.e. both NA and amino acids can hyperpolarize their target neuronal membrane, but the action of NA is not accompanied by an increase in ionic membrane conductance, as is the case for the amino acids GABA and glycine. The NA-induced inhibition of nerve cell activity in some brain areas mainly concerns neuronal maintained activity, while responses to electrical or sensory stimulation are either enhanced or left unaffected, at least in comparison to the maintained activity (Woodward et al., 1979; Moises and Woodward, 1980). It is attractive to suppose that such an action of NA on GABA-ergic transmission is also involved in controlling DPA-induced behaviour. Such an hypothesis explains why an active LC system is required for full expression of the DPA-evoked behaviour. The two areas in which GABA-ergic systems appear to be primarily involved in the induction of quasi-morphine abstinence behaviour, namely the Ac and the Cm-Pf, have been shown to receive projections from the LC (Ungerstedt, 1971; Emson et al., 1979; McGuiness and Krauthamer, 1980).

In the present experiments four structures were studied which are also involved in true morphine abstinence behaviour, as revealed with the technique of microinjection of antagonists (see section 1.3.3) namely, the Ac as a part of the amygdala, the Cm-Pf as a part of the medial thalamus, the nucleus caudatus as a part of the striatum and the LC as a part of the floor of the 4th ventricle. The data suggest that only in the Cm-Pf a GABA-ergic system may play a direct role in the expression of morphine withdrawal symptoms. In the amygdala and LC other neurotransmitter systems

are probably involved.

Conclusion

Administration of DPA to a rat results in quasi-morphine abstinence behaviour. In this thesis the mechanisms underlying this behaviour have been investigated further and attempts have been made to locate the site of action of DPA in initiating this behaviour. DPA acts via specific inhibition of the second enzyme of the GABA degradation pathway, SSA-DH, leading to an accumulation of SSA. This SSA probably enhances the level of GABA by prevention of further GABA-T catalysed conversion of GABA, or through backward formation of GABA. This enhanced GABA concentration, located in the nerve endings, raises GABA-ergic transmission, thus initiating quasi-abstinence behaviour.

Concomitantly increased GABA concentration in glial cells will suppress the behaviour through stimulation of presynaptic autoreceptors. Stimulation of autoreceptors following overflow of GABA to presynaptic areas may be responsible for the termination of quasi-morphine abstinence behaviour.

The increased GABA-ergic nerve cell activity probably initiates body shakes directly in the Cm-Pf or via disinhibition in the Ac. Stimulation of locomotor activity by DPA probably also involves a GABA-ergic system in the Cm-Pf. All events mentioned thus far will occur in the presence of a normally active LC. Taking into account the neuromodulatory action of NA, it may be suggested that, in this case, the neurons in the Ac and in the Cm-Pf are capable of differentiating between signal and noise, considering the enhanced GABA-ergic stimulation as such a signal. However, when the activity of the LC is decreased either through suppression by morphine or through degeneration of the projections to the Ac and the Cm-Pf, the cells in both nuclei might be less capable of differentiating signal from noise. As a result their capacity to evoke the signals required to initiate body shakes and enhanced locomotor activity would be decreased.

REFERENCES

Aghajanian, G.K. (1978) Tolerance of locus coeruleus neurons to morphine and suppression of withdrawal respons by clonidine. Nature 276, 186-188

Anlezark, G., Horton, R.W., Meldrum, B.S., Sawaya, M.C.B. (1976) Anticonvulsant action of ethanolamine-O-sulphate and di-n-propylacetate and the metabolism of γ-aminobutyric acid (GABA) in mice with audiogenic seizures. Biochem. Pharmacol. 25, 413-417

Ben-Ari, Y., Kanazawa, I., Zigmond, R.E. (1976) Regional distribution of

glutamate decarboxylase and GABA within the amygdaloid complex andstria terminalis system of the rat.J. Neurochem. 26, 1279-1283
Ben-Ari, Y., Lagowska, J., Tremblay, E., Le Gal La Salle, G. (1979) A new model of focal status epilepticus: intra-amygdaloid application of kainic acid elicits secondarily generalized convulsive seizures. Brain Res. 163, 176-179
Bird, S.J., Kuhar, M.J. (1977) Iontophorectic application of opiates to the locus coeruleus. Brain Res. 122, 523-533
Brennan, M.J.W., Cantrill, R.C., Oldfield, M., Krogsgaard-Larsen, P. (1981) Inhibition of γ-aminobutyric acid release by γ-aminobutyric acid-agonist drugs. Molec. Pharmacol. 19, 27-30
Corrigall, W.A., Linseman, M.A. (1980) A specific effect of morphine on evoked activity in the rat hippocampal slice. Brain Res. 192, 227-238
De Boer, Th. (1977) GABA shunt enzymes and the relationship with morphine abstinence. Academic Thesis, Erasmus University, Rotterdam, The Netherlands
Descarries, L., Watkins, K.C., Lapierre, Y. (1977) Noradrenergic axon terminals in the cerebral cortex of rat. III. Topometric ultrastructural analysis. Brain Res. 133, 197-222
Duffy, T.E., Nelson, S.R., Lowry, O.H. (1972) Cerebral carbohydrate metabolism during acute hypoxia and recovery. J. Neurochem. 19, 959-977
Emson, P.C., Björklund, A., Lindvall, O., Paxinos, G. (1979) Contributions of different afferent pathways to the catecholamine and 5-hydroxytryptamine innervation of the amygdala: a neurochemical and histochemical study. Neuroscience 4, 1347-1357
Fielding, S., Wilker, J., Hynes, M., Szewack, M., Novick Jr., W.J., Lal, H. (1978) A comparison of clonidine for antinociceptive and antiwithdrawal actions. J. Pharmacol. Exp. Ther. 207, 899-905
Godin, Y., Heiner, L., Mark, J., Mandel, P. (1969) Effects of di-n-propylacetate, an anticonvulsive compound, on GABA metabolism. J. Neurochem. 16, 869-873
Guyenet, P.G., Aghajanian, G.K. (1979) Ach, Substance P and Met-enkephalin in the locus coeruleus: pharmacological evidence for independent sites of action. Europ. J. Pharmacol. 53, 319-328
Harvey, P.K.P., Bradford, H.F., Davison, A.N. (1975) The inhibitory effect of sodium n-dipropylacetate on the degradative enzymes of the GABA-shunt. FEBS Lett. 52, 251-254
Herz, A., Bläsig, J., Papeschi, R. (1974) Role of catecholaminergic mechanisms in the expression of the morphine abstinence syndrome in rats. Psychopharmacologia 39, 121-143
Iadarola, M.J., Gale, K. (1979) Dissociation between drug-induced increases in nerve terminal and non-nerve terminal pools of GABA *in vivo*. Europ. J. Pharmac. 59, 125-129
Kuriyama, K., Yoneda, Y. (1978) Morphine-induced alterations of γ-aminobutyric acid and taurine contents and L-glutamate decarboxylase activity in rat spinal cord and thalamus: possible correlates with analgesic action of morphine. Brain Res. 148, 163-179
Le Gal La Salle, G., Paxinos, G., Emson, P., Ben-Ari, Y. (1978) Neurochemical mapping of GABA-ergic systems in the amygdaloid complex and bed nucleus of the stria terminalis. Brain Res. 155, 397-403
Massari, V.J., Gottesfeld, Z., Jacobowitz, D.M. (1976) Distribution of glutamic acid decarboxylase in certain rhombencephalic and thalamic nuclei of the rat. Brain Res. 118, 147-151
McGuiness, C.M., Krauthamer, G.M. (1980) The afferent projections to the centrum medianum of the cat as demonstrated by retrograde transport of horseradish peroxidase. Brain Res. 184, 255-269

Minchin, M.C.W., Iversen, L.L. (1974) Release of (^{3}H) gamma-aminobutyric acid from glial cells in rat dorsal root ganglia. J. Neurochemistry 23, 533-540
Mitchell, P.R., Martin, I.L. (1978) Is GABA release modulated by presynaptic receptors? Nature 274, 904-905
Moises, H.C., Woodward, D.J. (1980) Potentiation of GABA inhibitory action in cerebellum by locus coeruleus stimulation. Brain Res. 182, 327-344
Salganicoff, L., De Robertis, E. (1965) Subcellular distribution of the enzymes of the glutamic acid, glutamine and γ-aminobutyric acid cycles in rat brain. J. Neurochemistry 12, 287-309
Sarhan, S., Seiler, N. (1979) Metabolic inhibitors and subcellular distribution of GABA. J. Neurosci. Res. 4, 399-422
Sawaya, M.C.B., Horton, R.W., Meldrum, B.S. (1975) Effects of anticonvulsant drugs on the cerebral enzymes metabolizing GABA. Epilepsia 16, 649-655
Simler, S., Cieselski, L., Maitre, M., Randrianarisoa, H., Mandel, P. (1973) Effect of sodium n-dipropylacetate on audiogenic seizures and brain γ-aminobutyric acid level. Biochem. Pharmacol. 22, 1701-1708
Ticku, M.K., Huffman, R.D. (1980) The effects of acute and chronic morphine administration on GABA receptor binding. Europ. J. Pharmacol. 68, 97-106
Tzeng, S.F., Ho, I.K. (1978) Acute and continuous morphine administration on the γ-aminobutyric acid system in the mouse. Prog. Neuro-Psycho-pharmac. 2, 55-64
Ungerstedt, U. (1971) Stereotaxic mapping of the monoamine pathways in the rat brain. Acta Physiol. Scand. Suppl. 367, 1-48
Van der Heyden, J.A.M., Venema, K., Korf, J. (1980) *In vivo* release of endogenous GABA from rat striatum: effects of muscimol, oxotremorine and morphine. J. Neurochem. 34, 1648-1653
Woodward, D.J., Moises, H.C., Waterhouse, B.D., Hoffer, B.J., Freedman, R. (1979) Modulatory actions of norepinephrine in the central nervous system. Fed. Proc. 38, 2109-2116.

SUMMARY

Di-n-propylacetate (DPA), generally known to be an anti-epileptic drug, induces a behavioural syndrome in rats resembling morphine abstinence behaviour, which is called, therefore, quasi-morphine abstinence behaviour. An increase in GABA-ergic activity is probably responsible for this behavioural effect of DPA, since it has been shown to be suppressed by GABA-antagonists like bicuculline and picrotoxin.

The experiments presented in this thesis were designed to investigate the central mechanisms involved in this quasi-morphine abstinence behaviour.

In chapter I a survey is given of the biochemistry and physiology of GABA, and possible levels for regulation are indicated. In addition, the morphine withdrawal syndrome is described briefly. A lot of compounds have been reported to induce quasi-abstinence behaviour, similar to a greater or lesser extent to that induced by DPA. The implication of these data, with respect to the mechanisms involved in morphine abstinence behaviour, are discussed. Finally, the interactions between GABA and opioids are reviewed. The data suggest that such interactions exist at the cellular level.

In chapter II studies on the mechanism of action of DPA are described, particularly the effect of DPA on the enzymes involved in the degradation of GABA. It appears that DPA increases GABA-ergic activity in the brain via inhibition of the second enzyme involved in GABA-degradation, viz. succinic semialdehyde dehydrogenase (SSA-DH). DPA does not exert a direct effect on the first enzyme GABA-transaminase (GABA-T), as suggested by other authors. Inhibition of SSA-DH will result in accumulation of SSA, which probably inhibits GABA-T, thus increasing the concentration of GABA.

In the experiments described in chapter III, two branched-chain fatty acid analogues of DPA and a short-chain fatty acid were used to study the relationship between their biochemical and behavioural effects. A correlation appeared to exist between the K_i of these compounds for SSA-DH and the doses exerting a maximum effect on behaviour. On the other hand, concurrent inhibition of GABA-T seemed to suppress the behavioural effects of the fatty acids. This apparent paradox can possibly be explained by supposing a different action of the fatty acids in distinct compartments of the brain, suggesting that the increased GABA-ergic activity in the neuronal compartment is evoked by SSA-DH inhibition and initiates quasi-morphine abstinence behaviour. A concomitant increase in the GABA concentration in glial cells, caused by inhibition of GABA-T, is held responsible for the reduced

capability of the fatty acids to induce the behavioural syndrome when compared with DPA.

The time course of the DPA-induced behaviour is much shorter than that of the DPA-induced enhancement of the GABA-concentration in brain. Therefore, as described in chapter IV, we have studied the influence of enhanced GABA-levels, caused by a first injection of DPA, on the behaviour evoked by a second injection of DPA at different time intervals after the first injection. The results indicate that GABA fulfils a role both in the initiation and termination of DPA-induced behaviour. The mechanism responsible for this dual action of GABA is ascribed to a different sensitivity to DPA of the nerve terminal and glial metabolic compartments of GABA in the brain, as suggested above. The increase in GABA in the nerve terminal caused by DPA is probably responsible for the initiation of the quasi-abstinence behaviour, whereas the overflow of GABA from the synaptic cleft to presynaptic areas may be responsible for the suppression of this behaviour via stimulation of autoreceptors.

Behavioural responses after intracerebral injections of DPA were studied to obtain information on the brain areas involved in the quasi-abstinence syndrome (Chapters V and VI). The results suggest a facilitatory role for a GABA-ergic system in the centre median-parafascicularis area on body shakes, while in the central amygdala a GABA-ergic system appears to exert an inhibitory influence on this symptom of abstinence. The GABA-ergic terminals present in the locus coeruleus do not seem to play a role in this behaviour. On the other hand, stimulation of opiate receptors in the latter nucleus by low doses of morphine, thus decreasing the activity of the noradrenergic cells, was found to suppress this behaviour almost totally. The centre median-parafascicularis area is much less sensitive to this suppressive effect of morphine, while no suppression by morphine could be found when injected into the central amygdala. A decrease in locus coeruleus activity by destruction of the cells or by degeneration of the projections to the forebrain by about 80% prevented the full expression of the quasi-abstinence behaviour.

Thus, an active locus coeruleus is required for the induction of DPA-induced abstinence behaviour. The role of the locus coeruleus can possibly be understood in the light of a neuromodulatory function of noradrenaline on GABA-ergic inhibition.

It is concluded that quasi-morphine abstinence behaviour is evoked by DPA through enhancement of GABA-ergic transmission in certain areas of the brain, such as the centre median-parafascicularis area, or via disinhibition of neurons lying in the central amygdala. An active noradrenergic system is a prerequisite for an efficient transmission of the GABA-ergic signals in these nuclei.

SAMENVATTING

KWASI-MORFINE ONTHOUDINGSGEDRAG OPGEWEKT DOOR DIPROPYLACETAAT IN DE RAT: MECHANISMEN EN AANGRIJPINGSPUNTEN

De opmerkelijke waarneming dat dipropylacetaat (Depakine^{R}) bij ratten een gedrag opwekt dat veel overeenkomsten vertoont met het patroon van gedragssymptomen dat optreedt na het onthouden van morfine aan verslaafde ratten, is aanleiding geweest voor ons onderzoek. We duiden dit gedrag ook wel aan als kwasi-morfine onthoudingsgedrag. Nader onderzoek van dit gedrag zou informatie kunnen leveren over de processen die optreden bij het echte morfine onthoudingsgedrag. De belangrijkste symptomen van het door dipropylacetaat (DPA) geïnduceerde gedrag bij de rat zijn: het schudden van het hele lichaam zoals door een natte hond, het graven in het zaagsel van de observatiekooi, een hoge rug en rechtopstaande haren en een sterk verhoogde bewegingsaktiviteit.

Het was bekend uit de literatuur dat DPA de hoeveelheid gamma-aminoboterzuur (GABA) in de hersenen verhoogt. GABA verzorgt in de hersenen een functie als overdrachtsstof (transmitter) tussen zenuwcellen (neuronen) en komt voor in nagenoeg alle delen van de hersenen en het ruggemerg. Uit het onderzoek van De Boer en Bruinvels, dat aan het in dit proefschrift beschreven onderzoek voorafging, is dan ook gebleken dat de werking van de neuronen die GABA bevatten, gestimuleerd wordt tijdens het door DPA opgewekte gedrag. In dit proefschrift is beschreven hoe DPA deze verhoogde stimulatie door GABA veroorzaakt en ook waar DPA in de hersenen aangrijpt.

In de inleiding (hoofdstuk I) is aangegeven dat de aktiviteit van het GABA-erge systeem op verschillende niveaus beinvloed kan worden, nl. op het niveau van de aanmaak en afbraak van GABA, van de mate van GABA-afgifte door de GABA-erge zenuwuiteinden en van de gevoeligheid voor GABA van de ontvangercellen. Omdat DPA veel wordt gebruikt als geneesmiddel bij epilepsie, is ook kort ingegaan op de mechanismen die aan deze anti-epileptische werking van DPA ten grondslag kunnen liggen. Met betrekking tot het morfine onthoudingsgedrag is een overzicht gegeven van wat er tot dusver is bestudeerd aan kwasi-onthoudingsgedrag. Behalve door DPA kan dit gedrag namelijk nog door veel andere stoffen worden opgewekt. Hoewel er duidelijke verschillen zijn aan te geven tussen de syndromen die door verschillende klassen van stoffen opgewekt worden, is gebleken dat de informatie, verkregen uit zulk onderzoek, een werkelijke bijdrage levert tot de ontrafeling van de mechanismen die een rol

spelen bij morfine-onthoudingsgedrag. In het laatste gedeelte van de inleiding is nagegaan of de literatuur-gegevens over de interacties tussen morfine en GABA in overeenstemming zijn met de rol van GABA die wordt gesuggereerd door de gegevens omtrent het door DPA opgewekte onthoudingsgedrag.

Ons eerste onderzoek (hoofdstuk II) betrof het mechanisme waardoor DPA de hoeveelheid GABA in de hersenen verhoogt. Deze verhoging komt tot stand door remming van de afbraak van GABA. Twee specifieke enzymen zijn bij deze afbraak betrokken, achtereenvolgens GABA-transaminase (GABA-T) en succinaat semi-aldehyde dehydrogenase (SSA-DH) (zie Fig. 1).

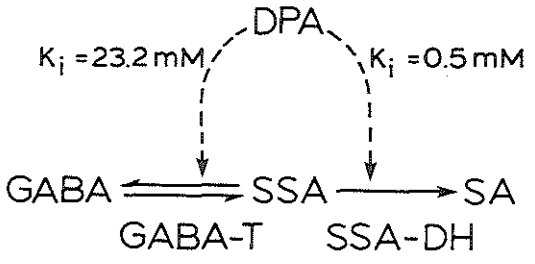

FIG. 1. Effect van DPA op de afbraak van GABA. SSA = succinaat semi-aldehyde; SA = succinaat. De K_i is een maat voor het remmend effect van een stof. Een lagere K_i wil zeggen een sterkere remming.

In hoofdstuk II is beschreven dat DPA het tweede enzym 30-40 maal sterker remt dan het eerste, waardoor ophoping plaatsvindt van SSA. Omdat GABA-T geremd wordt door het tussenprodukt SSA, is het uiteindelijk resultaat van de werking van DPA ophoping van GABA.

Ligt dit mechanisme nu ten grondslag aan het kwasi-morfine onthoudingsgedrag? Voor de beantwoording van deze vraag hebben we gebruik gemaakt van op DPA gelijkende stoffen. Door van al deze stoffen te vergelijken wat hun effect was op het gedrag en op de afbraak van GABA, konden we dit inderdaad aannemelijk maken. Het bleek dat de dosis van deze stoffen waarmee het maximale effect op het gedrag bereikt kan worden, lager is naarmate SSA-DH sterker geremd wordt. De mate waarin het gedrag wordt opgewekt is echter gering voor die stoffen die tegelijk een sterk remmend effect hebben op GABA-T. We hebben dit verklaard door aan te nemen dat de verschillende cellen waarin de afbraak van GABA plaatsvindt, nl. de zenuwceluiteinden en de gliacellen (die een opbouw- en onderhoudsfunctie in de hersenen vervullen), enzymen bevatten die een verschillende gevoeligheid hebben voor deze stoffen. Gegevens uit de literatuur ondersteunen deze aanname. Remming van GABA-T

vindt plaats in de gliacellen en in de ontvangercellen, terwijl remming van SSA-DH plaatsvindt in de zenuwceluiteinden van waaruit GABA ook wordt afgegeven. Deze afgifte is proces 1 in Fig. 2. Ophoping van GABA in de gliacellen resulteert echter ook in afgifte van GABA (proces 2), met als gevolg dat GABA aangrijpingspunten (receptoren) op het zenuwuiteinde bereikt (R_2), die zorgen voor een terugkoppeling op de afgifte van GABA (proces 4). Aangezien verhoging van deze afgifte van GABA verantwoordelijk is voor het kwasi-onthoudingsgedrag, zal remming van deze afgifte dit gedrag doen afnemen.

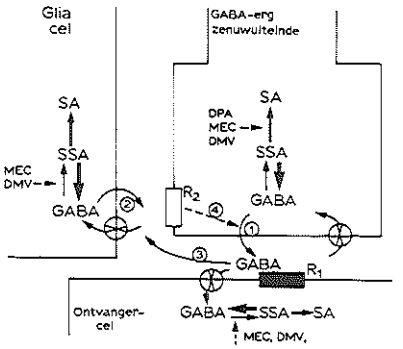

FIG. 2. Effect van DPA en soortgelijke stoffen, methylethylcaproaat (MEC) en dimethylvaleraat (DMV) op de afbraak van GABA in de onderscheiden soorten cellen in de hersenen. MEC en DMV remmen GABA-T in gliacel en ontvangercel, terwijl beide stoffen, evenals DPA, SSA-DH remmen in het GABA-erge zenuwuiteinde. Voor verklaring van de cijfers zie de tekst. Ø-opnamemechanisme voor GABA, waarmee de werking van GABA wordt beeindigd. R1 - aangrijpingspunt voor GABA op ontvangercel. R2 - aangrijpingsplaats voor GABA op het GABA-erge zenuwuiteinde van waaruit een terugkoppeling op de afgifte van GABA wordt gegeven.

In een volgende studie (hoofdstuk IV) is beschreven dat een dergelijk tweeledig mechanisme ook een rol kan spelen bij de beëindiging van het door DPA opgewekte gedrag, die bij de meest gebruikte dosering (300 mg/kg, ingespoten in de buikholte) optreedt na 10-15 minuten. Wanneer DPA voor een tweede keer werd geinjecteerd, korte tijd (20 min - 4 uur) na de eerste injectie, kwam het kwasi-abstinentie gedrag slechts gedeeltelijk tot uiting. Er kon een verband worden gelegd tussen de verhoging van de GABA-concentratie in de hersenen door de eerste DPA-injectie (zie Fig. 3) en de remming van het onthoudingsgedrag. Wanneer deze verhoging maximaal was, werd ook het onthoudingsgedrag het sterkst geremd. Experimenten met hogere doseringen van DPA ondersteunden de suggestie dat GABA een tweeledige rol vervult in het kwasi-onthoudingsgedrag.

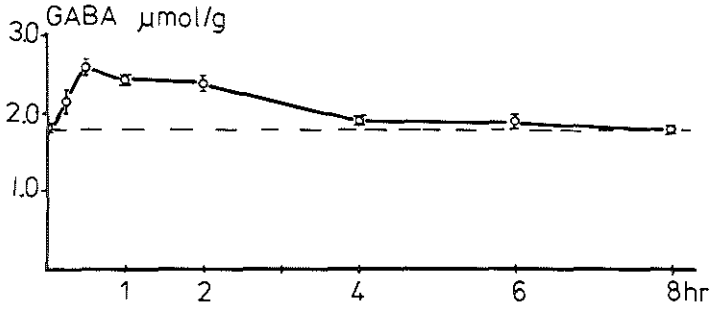

FIG. 3. Verloop van de hoeveelheid GABA in de hersenen van ratten na een orale toediening DPA (400 mg/kg). Naar Kukino en Deguchi (1977).

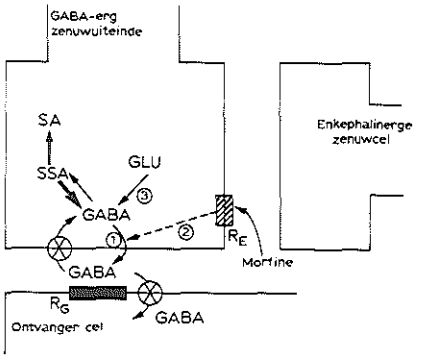

FIG. 4. Effect van stimulering van een enkefaline/endorfine receptor (R_E) (bijv. door morfine) op de afgifte van GABA. 1 = Afgifte van GABA; 2 = remming van de afgifte; 3 = synthese van GABA. R_G is aangrijpingsplaats voor GABA.

Het mechanisme, waarmee DPA het kwasi-abstinentie gedrag opwekt en even later onderdrukt, stellen we ons dan als volgt voor: DPA remt SSA-DH in het zenuwuiteinde en veroorzaakt zo ophoping van GABA. Deze verhoogde concentratie leidt ook tot verhoogde afgifte van GABA en induceert via verder nog onbekende mechanismen het kwasi-onthoudingsgedrag. De systemen voor opname van GABA in de omringende cellen, die een eind moeten maken aan de werking van GABA, zijn door deze verhoogde concentratie van GABA echter niet meer in staat om de GABA zo snel op te nemen. GABA bereikt daardoor (proces 3 in Fig. 2) de plaatsen op het zenuwceluiteinde, van waaruit het een terugkoppeling geeft op de afgifte van GABA uit ditzelfde uiteinde. Dit laatste proces leidt er dan toe dat het kwasi-onthoudingsgedrag beëindigd wordt.

Het is aantrekkelijk om nu te veronderstellen dat een dergelijke verhoogde aktiviteit van GABA-erge neuronen ook een rol speelt tijdens echte morfine onthouding. In de literatuur wordt de wisselwerking tussen morfine en GABA vooral gezien als een onderdrukking van de GABA-afgifte uit zenuwuiteinden door morfine via interactie met de aangrijpingsplaats van enkefaline of endorfine (als morfine werkzame, lichaamseigen stoffen) (proces 2, Fig. 4). Ook de synthese (proces 3) van GABA wordt waarschijnlijk geremd door morfine. De veronderstelling dat dit kan leiden tot een verhoogde GABA-erge aktiviteit tijdens morfine onthouding moet nog experimenteel bevestigd worden.

Naast de vraag naar <u>hoe</u> vonden we het ook belangrijk om te weten <u>waar</u> DPA in de hersenen het kwasi-onthoudingsgedrag opwekt. In hoofdstuk V is beschreven dat twee gebieden, nl. de "centre median-parafascicularis" en de centrale amygdala (zie Fig. 5) allereerst betrokken lijken te zijn.

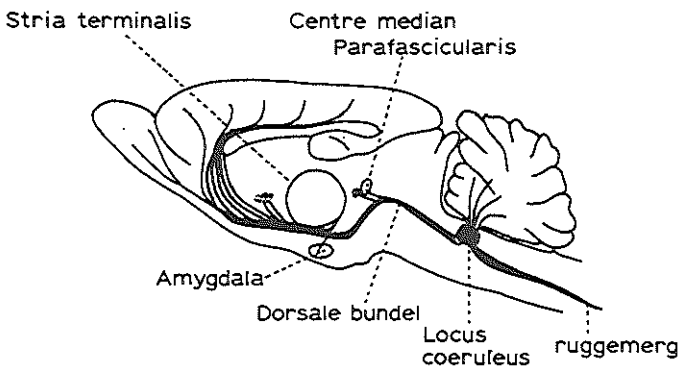

FIG. 5. Overlangse doorsnede van de hersenen van een rat. Met de zwarte lijnen zijn de zenuwbanen die ontspringen in de locus coeruleus, aangegeven.

Het GABA-erge systeem in de centre median-parafascicularis lijkt een aangrijpingspunt voor DPA te zijn, omdat injectie van DPA ter plaatse resulteerde in het natte hond syndroom. GABA-tegenwerkende stoffen alsook morfine onderdrukken dit gedrag wanneer ze in de centre median-parafascicularis worden geinjecteerd 5 minuten nadat DPA door injectie in de buik is toegediend. Aan de andere kant bleek het GABA-erge systeem in de centrale amygdala juist een remmende invloed uit te oefenen op het door DPA geinduceerde gedrag. Desondanks resulteerde beschadiging van dit laatste gebied in vermindering van het natte hond syndroom. De centrale amygdala lijkt dus niet een direkt aangrijpingspunt voor DPA te zijn, maar vervult wel een belangrijke rol in het kwasi-onthoudingsgedrag.

De studie beschreven in hoofdstuk VI bracht aan het licht dat nog een derde hersenstruktuur, nl. de locus coeruleus, onmisbaar is voor de gedragseffecten van DPA. Van de locus coeruleus is bekend dat het wijd vertakte uitlopers heeft naar bijna alle delen van de hersenen (zie Fig. 5). Het gebied bleek met name heel gevoelig te zijn voor morfine, dat de aktiviteit van de zenuwcellen in de locus coeruleus onderdrukt. Een 25 keer lagere dosering van morfine dan gebruikt was voor de centre median-parafascicularis was in staat, na injectie in de locus coeruleus, het kwasi-onthoudingsgedrag nagenoeg volledig te onderdrukken. Ook uitschakeling van deze zenuwcellen op andere manieren verminderde het door DPA geinduceerde gedrag aanzienlijk. Een mogelijke verklaring voor de noodzakelijke aanwezigheid van de locus coeruleus is dat de overdrachtsstof, waarvan zich de cellen van deze kern bedienen, de werking van GABA potentieert, waardoor de informatieoverdracht van GABA-erge cellen op ontvangercellen efficienter verloopt. Blijkbaar is deze potentiering zo zeer noodzakelijk dat zonder de aktiviteit van de locus coeruleus de stimulering van GABA door DPA in bijvoorbeeld de centre median-parafascicularis niet kan resulteren in kwasi-morfine onthoudingsgedrag.

Samengevat luiden de conclusies van de in dit proefschrift beschreven studies als volgt:

1. Hoe wekt DPA kwasi-onthoudingsgedrag op? DPA remt de afbraak van GABA door remming van het tweede enzym in de afbraak, SSA-DH.
2. Waar ligt het aangrijpingspunt van DPA? Voor het natte hond syndroom waarschijnlijk in de centre median-parafascicularis, terwijl een intakte centrale amygdala en locus coeruleus onmisbaar lijken te zijn.

Curriculum vitae

Op 21 december 1952 werd ik geboren te Groningen en na de lagere school en het Gereformeerd Lyceum in deze plaats doorlopen te hebben, behaalde ik in 1970 mijn diploma HBS-B. Daarna werd gestart met de studie scheikunde aan de Rijksuniversiteit van Groningen. Na in mei 1973 geslaagd te zijn voor het kandidaatsexamen, koos ik als specialisatie de biochemie. Het hoofdvakonderzoek over bacteriële membranen werd verricht onder leiding van Prof. Dr. B. Witholt. Dr. J. Korf was begeleider bij het bijvak-onderzoek in de biologische psychiatrie. Na het doctoraal examen in april 1977 trad ik in dienst van de Nederlandse Stichting voor Zuiver-Wetenschappelijk Onderzoek ten behoeve van de stichting FUNGO. Het onderzoek vond plaats op de afdeling Farmacologie van de Erasmus Universiteit te Rotterdam onder supervisie van Prof. Dr. J. Bruinvels, en leidde tot dit proefschrift. In dit onderzoek werd ook samengewerkt met Dr. A.R. Cools van het Instituut voor Farmacologie van de Katholieke Universiteit te Nijmegen. Vanaf begin 1980 ben ik in dienst van het Rijks Instituut voor de Volksgezondheid te Bilthoven en werkzaam op het Laboratorium voor Farmacologie (afdeling Psychofarmacologie) te Utrecht.