$GABA_B$ Receptor Modulation of Ethanol-Stimulated Locomotor Activity

By

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CERTIFICATE OF APPROVAL

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Associate Dean for Graduate Studies

DEDICATION

I would like to dedicate this dissertation to my wife Michele, and son Noah.
Thank you for your unwavering love and support.

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LIST OF ABBREVIATIONS

αCSF – Artificial Cerebrospinal Fluid

ACT – Activity

AVTA – Anterior Ventral Tegmental Area

Bacl - Baclofen

CA1 – Field of Hippocampus

CA3 – Field of Hippocampus

CON – Non-Selected Control Mouse Line

depr – Locomotor Depression

DA - dopamine

EtOH – Ethanol/Alcohol

FAST – Selected Mouse Line Sensitive to Ethanol Stimulation

FH+ - Family History Positive

FH- - Family History Negative

GABA – γ-Aminobutyric Acid

Glu - Glutamate

Hipp – Hippocampus

ICV - Intracerebroventricular

IP - Intraperitoneal

NAcc – Nucleus Accumbens

PAG – Periaquiductal Gray

Pfcx – Prefrontal Cortex

PVTA – Posterior Ventral Tegmental Area

QTL – Quantitative Trait Locus (Loci)

RMC – Red Nucleus Magnocellular

RPC – Red Nucleus Parvocellular

SLOW – Selected Mouse Line Insensitive to Ethanol Stimulation

SNC – Substantia Nigra Pars Compacta

SNR – Substantia Nigra Pars Reticulata

SOA – Sons of Alcoholic Fathers

SONA – Sons of Non-Alcoholic Fathers

stim – Locomotor Stimulation

VP/SI – Ventral Pallidum/Substantia Innominata

VTA – Ventral Tegmental Area

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None of my scientific achievements would have been possible had it not been for the support and guidance of my mentor, Dr. Tamara Phillips. I transferred into Tamara's laboratory from Dr. John Crabbe's laboratory. At the time, I had just completed all the requirements for advancement to candidacy for the PhD. My dissertation proposal involved assessing $GABA_B$ receptor modulation of EtOH's locomotor stimulant effects in the FAST and SLOW selected mouse lines, a direction that was more in line with Tamara's work. Tamara welcomed me into her laboratory without hesitation. Tamara is an excellent teacher, an incredible role model, and has become a good friend. Wherever my professional life takes me, I will have gotten there because of the mentoring experience I enjoyed working with Tamara.

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have become good friends, and I will miss them. Their input and support has meant everything. I can't imagine working with a better group of people.

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ABSTRACT

Sensitivity to the locomotor stimulant effects of ethanol (EtOH) may represent a mouse model of EtOH-induced euphoria in humans. Identifying neural substrates that mediate this EtOH effect may elucidate mechanisms influencing risk for alcohol abuse. In previous studies, systemic injection of baclofen, a γ-aminobutyric acid B ($GABA_B$) receptor agonist, attenuated EtOH's stimulant effects in FAST mice, selectively bred for extreme sensitivity to this effect. $GABA_B$ receptors on dopamine cell bodies in the ventral tegmental area (VTA) are believed to modulate EtOH-induced dopamine release. However, $GABA_B$ receptors are also located peripherally. Studies in which baclofen was injected intracerebroventricularly (ICV) or directly into the VTA of FAST mice were performed to test the hypotheses that 1) central $GABA_B$ receptors mediate baclofen's effects on EtOH-stimulated activity, and 2) VTA $GABA_B$ receptors specifically modulate EtOH's stimulant effects. ICV baclofen dose-dependently attenuated EtOH's locomotor stimulant effects, suggesting a central locus for baclofen's effects. Anterior, intra-VTA baclofen attenuated EtOH's stimulant effects, whereas posterior injection potentiated these effects. Mice bred for resistance to EtOH's stimulant effects (SLOW mice) were also tested. Anterior intra-VTA baclofen alone had a locomotor sedative effect not seen in FAST mice, whereas posterior intra-VTA baclofen had no effect. These results support a region-specific role for VTA $GABA_B$ receptors in modulation of EtOH's locomotor stimulant effects. Furthermore, selection for differential EtOH stimulant sensitivity appears to have altered VTA $GABA_B$ systems that influence locomotor behavior. $GABA_B$ receptors are proposed as a target for modulation of EtOH's euphoric effects.

An intriguing finding from the above microinjection study was that anterior intra-VTA baclofen produced a greater locomotor sedative effect in SLOW mice than in FAST mice. These results suggested that compared to FAST mice, $GABA_B$ receptor density in the anterior region of the VTA might have been increased by genetic selection in SLOW mice. Thus, we examined the VTA of FAST and SLOW mice using receptor autoradiography to determine the density and distribution of $GABA_B$ receptors. Results showed that neither anterior nor posterior $GABA_B$ receptor densities differed between FAST and SLOW mice. Moreover, when a similar analysis was conducted for the SNC, a motor control structure located near the VTA, no significant line difference in anterior or posterior $GABA_B$ receptor density was found. These data do not support the hypothesis that genetic selection for differential sensitivity to EtOH's locomotor stimulant effects resulted in increased anterior VTA $GABA_B$ receptor density in SLOW, relative to FAST mice. Furthermore, because a line difference in $GABA_B$ receptor density was not found in the SNC, our attention was not refocused onto this structure as a possible mediator of the line difference in baclofen sensitivity, although its involvement cannot be ruled out. Based on current data, we hypothesize that genetic selection of the FAST and SLOW lines differentially altered anterior VTA $GABA_B$ receptor subunit composition or function, or perhaps the intracellular signaling of a neural system distinct from, but influenced by $GABA_B$ receptor activation. The possibility of SNC $GABA_B$ receptor involvement in the mediation of our behavioral effects, as well as the possible involvement of $GABA_B$ receptors located elsewhere, will require further investigation.

INTRODUCTION

Alcohol (ethanol; EtOH) abuse and dependence is a major problem in the United States. A projected 30.8 million Americans exhibited signs of EtOH abuse or dependence in the year 2000 as outlined by Diagnostic and Statistical Manual-III criteria (Williams et al., 1989). However, this estimate does not reflect the total number of individuals consuming EtOH in the United States. Many more Americans casually consume alcoholic beverages. In 1998, per capita EtOH consumption of United States citizens aged 14 or older (based on the sales of beer, wine, and spirits) was 2.19 gallons (Nephew et al., 1998). Health problems stemming from this high level of EtOH consumption have been quite costly, both monetarily, and in terms of lives lost. Management of EtOH abuse and dependence issues in the United States cost an estimated $166.5 billion in 1995 (National Institute on Drug Abuse and National Institute on EtOH Abuse and Alcoholism, 1998). Furthermore, EtOH-related cirrhosis of the liver claimed an estimated 3.8 lives per 100,000 in 1997 (Saadatmand et al., 2000), and 12,663 persons lost their lives in EtOH-related traffic accidents in 1998 - 30.5% of all traffic fatalities in the United States were EtOH-related (Yi et al., 1999). Thus, research aimed at identifying prevention and treatment strategies for EtOH abuse and dependence is certainly warranted.

Genetic Risk Factors

The biological and environmental factors influencing EtOH abuse and dependence in humans are not well understood. However, twin, adoption, and family

studies suggest that genetic factors play an important role (Ball and Murry, 1994; Devor and Cloninger, 1989; Goodwin, 1985; Schuckit, 1987). These studies have lead to more recent work in which researchers have begun to identify putative genetic markers for alcoholism (Devor and Cloninger, 1989; Ferguson and Goldberg, 1997). A marker is a trait or gene product that is associated with sensitivity to EtOH's actions, or that is closely linked with alcoholism. A popular approach in identifying such markers has been to compare sons of alcoholic fathers (SOA) to sons of non-alcoholic fathers (SONA). Because EtOH abuse and dependence has a genetic component, SOA possess a greater risk of developing alcoholism than SONA.

A number of studies suggest that SOA are less sensitive to the effects of EtOH than are SONA. In a study of Danish subjects, SOA reported lower levels of nausea, dizziness, warmth, headache, palpitations, and general EtOH intoxication than SONA following administration of 0.5 g/kg 95% EtOH (Pollock et al., 1986). Importantly, self-reports of previous EtOH consumption and blood EtOH levels determined during the study argue against greater metabolic tolerance having developed in the SOA tested.

Similar results were seen in a population of American subjects (Schuckit, 1988). Compared to controls, SOA reported lower subjective feelings of intoxication and less intense body sway following ingestion of either 0.75 or 1.1 ml/kg 95% EtOH. Moreover, as reported in the Danish study, blood EtOH levels did not differ between the groups, again eliminating metabolic tolerance as an explanation for the results. Schuckit (1994) later published a follow-up study in which the same SOA were re-examined, and sub-divided into groups based on whether they had been diagnosed with alcoholism or not. Interestingly, ten years after the initial investigation, it was found that the subgroup of

high-risk individuals that subsequently developed alcoholism had reported lower subjective ratings of intoxication, and lower levels of EtOH-induced body sway than the subgroup that did not. The rate of alcoholism was greater in SOA than in SONA, however, among the low-risk individuals, those that reported lower sensitivity to EtOH in the initial investigation were more likely to have developed alcoholism. Taken together, these studies suggest that individuals who are at greater genetic risk for developing EtOH abuse disorders are less sensitive to EtOH's effects, and that EtOH sensitivity even predicts risk in those without a family history of alcoholism. Recent reports by Schuckit and Smith (2000), and Heath et al. (2001) have reached similar conclusions.

Not all studies support the above conclusion. In a study by Nagoshi and Wilson (1987), subjects that had an alcoholic first-degree relative (FH+) reported greater sensitivity to EtOH's intoxicating effects than subjects that did not (FH-). Blood EtOH levels were maintained at 100 mg/dl by ingestion of an initial alcoholic beverage containing 0.8 g/kg EtOH, followed by subsequent maintenance beverages. In another study, O'Malley and Maisto (1985) reported that despite comparable blood EtOH levels, FH+ subjects exhibited greater impairment than FH- subjects on a timed motor task. In the same study, FH- subjects reported higher levels of intoxication, behavioral impairment, anesthesia, and central stimulation. Finally, a third study showed that at blood EtOH levels of 63 mg/dl, FH+ subjects were more impaired than FH- subjects on the bead stringing and the hand steadiness motor tasks (Vogel-Sprott and Chipperfield, 1987). However, FH+ and FH- subjects did not differ in their subjective ratings of intoxication. Thus, the existing data are in disagreement about whether FH+ subjects are

indeed less sensitive to EtOH than FH- subjects. However, relative EtOH sensitivity between these risk groups may depend on the specific EtOH effect measured.

Though the above data appear contradictory, they show how measures of acute sensitivity to EtOH can be used to glean important information about subsequent risk for developing alcoholism. Furthermore, because these traits may represent biological trait markers for the development of alcoholism, understanding the mechanisms that influence EtOH's acute effects may ultimately aid in our understanding of how EtOH use disorders develop.

The Locomotor Stimulant Effects of EtOH: Human Studies

Acute administration of EtOH has a number of effects in humans. One of these is locomotor stimulation, and sensitivity to this EtOH effect may represent a biological trait marker for risk of developing EtOH abuse or alcoholism. In support of this contention, Newlin and Thomson (1991) showed that SOA exhibited greater motor stimulation than SONA following an acute administration of EtOH. Similar results have more recently been reported (Holdstock et al., 2000). However, in one study, the opposite result was seen, with greater stimulation correlating with a lower incidence of dependence in the male subjects tested (Poikolainen, 2000). Nevertheless, it is believed that the biological processes that mediate EtOH's locomotor stimulant properties may be the same biological processes that ultimately influence the development of EtOH use disorders.

Evidence suggests that the psychomotor stimulant effects of EtOH parallel the euphoric effects of the drug. Two studies by Ekman and collegues (1963; 1964) showed that following a single moderate dose of EtOH, subjects reported feeling more talkative

(stimulated – interpreted as a measure of locomotor stimulation), elated (euphoric), happy, and hazy. Furthermore, these effects occurred during the absorption phase of the blood EtOH curve. Similar findings were reported by Ahlenius et al. (1973), however, blood EtOH levels were not measured. In another study, subjects reported increased subjective euphoria and increased electroencephalogram alpha activity following consumption of 0.695 g/kg EtOH, and these effects were also observed during the absorption phase of the blood EtOH curve (Lukas and Mendelson, 1988). Taken together, these studies suggest that the locomotor stimulant and euphoric effects of EtOH occur during the ascending limb of the time-blood EtOH concentration curve. In support of this contention, Babor et al. (1983) concluded that EtOH's effects on affect are biphasic. That is, whereas positive affect (euphoria, and associated locomotor stimulation) is experienced during the rising phase of the time-blood EtOH concentration curve, negative affect (fatigue and depression) is experienced during the descending limb of the same curve.

The Locomotor Stimulant Effects of Ethanol: Animal Studies

EtOH-induced locomotor stimulation has also been studied in rodents. In one of the first reports on this EtOH effect, Read and colleagues (1960) tested Swiss-Webster mice on an apparatus consisting of a light plastic container (12 cm in diameter, and 12 cm high) supported on a spring lever. Movement within the container closed a microswitch so that locomotor activity could be measured. Following injection of 5 ml/kg EtOH (EtOH concentration not provided), mice exhibited a 300% increase in locomotion that returned to near basal levels 20 min post-injection. Moreover, the 20-min period of

locomotor stimulation was replaced with pronounced locomotor depression or sedation, suggesting that the biphasic effects of EtOH seen in human subjects are also seen in mice.

Numerous other studies have reported locomotor stimulant responses to acute EtOH administration in rodents. Jarbe and Ohlin (1977) injected Mongolian gerbils with 1 or 2 g/kg EtOH (10% w/v) and immediately tested them in a square-shaped open-field for 5 min. The arena was divided into 16 smaller squares so that movement from square to square could be quantified as a measure of locomotion. EtOH dose-dependently stimulated open-field activity among the gerbils tested. Similar results were seen in albino rats (Buckalew and Cartwright, 1968), where EtOH doses up to 1.2 g/kg (30 % v/v) resulted in marked increases in exploratory behavior in an operant chamber, whereas EtOH doses greater than 1.8 g/kg reduced exploratory behavior.

Taken together, the results of the above human and rodent studies, along with many additional studies in rodents, suggest that the biphasic effects of EtOH on locomotor behavior are dependent on both EtOH dose, and time after EtOH administration (Pohorecky, 1977; Dudek et al., 1991). These studies suggest that EtOH stimulates locomotion at lower doses, but has locomotor depressant or sedative effects at higher doses. Furthermore, EtOH increases locomotion at early time points after EtOH administration, during the ascending limb of the time-blood EtOH concentration curve, but decreases locomotion at time points on the descending limb of the time-blood EtOH concentration curve. However, it should be noted that not all studies have shown an EtOH-induced locomotor stimulant response in rats and mice (Frye and Breese, 1981; Crabbe et al., 1986; Masur et al., 1986; Cunningham et al., 1992; Cunningham et al., 1993). The reason for these disparate findings are not well understood, but may be

related to differing testing conditions across laboratories (Crabbe et al., 1988; Crabbe et al., 1999), or genetic differences (Crabbe et al., 1982; Crabbe, 1986; Crabbe et al., 1994; Cunningham et al., 1992; Dudek and Tritto, 1994; Phillips et al., 1995), a possibility that I will return to in subsequent sections.

Significance of the Locomotor Stimulant Effects of EtOH

Several studies suggest that EtOH's rewarding properties are also biphasic in nature. In one study, mice from the DBA/2J inbred strain were tested in an EtOH conditioned place preference paradigm in which a moderate dose of EtOH (3 g/kg, concentration not given) was paired with one floor, and saline injection was paired with the other (Risinger and Cunningham, 1992). Three separate groups of mice received drug-floor pairings separated by either no delay after injection, a 30 min delay, or a 60 min delay. Following the training phase, mice were administered a drug-free test trial. Mice having experienced the pairings separated by no delay after injection spent more time on the floor that had been paired with EtOH (conditioned place preference). However, mice that had been trained with the 30-min delay spent more time on the saline-paired floor (conditioned place aversion). Thus, if training occurred while EtOH concentrations were rising (no delay), mice found EtOH's effects rewarding. However, if training occurred after a 30 min delay, when blood EtOH levels had leveled off, or were descending, mice found EtOH's effects aversive. In a related study, Cunningham and Prather (1992) reported that, compared to 15- and 30-min conditioning trial durations, DBA/2J mice developed greater EtOH-conditioned place preference to 2 g/kg (20% v/v) EtOH injection when 5-min conditioning trial durations were employed, suggesting that

EtOH was more reinforcing at early time points following administration. Finally, in a more recent report, Lewis and June (1990) showed that EtOH doses ranging from 0.10 to 1.5 g/kg (EtOH concentration not given) reduced the threshold and increased the response rates for brain stimulation reward, but only during the ascending limb of the time-blood EtOH concentration curve. Together, these data suggest that EtOH's rewarding effects, similar to its locomotor stimulant effects, also depend on the dose of EtOH, as well as time after EtOH administration.

The dose- and time-dependent relationship between EtOH's locomotor stimulant and rewarding effects has led researchers to speculate that EtOH stimulation in humans may reflect its rewarding and euphoric effects. Moreover, sensitivity to EtOH-induced locomotor stimulation in rodents may represent an animal model of EtOH's rewarding and/or euphoric effects. Formulated in their psychomotor stimulant theory of addiction, Wise and Bozarth (1987) argued that all drugs that are positive reinforcers not only activate the midbrain mesolimbic dopamine projection, but also induce forward locomotion toward novel or salient stimuli. Thus, these authors suggested that both the locomotor stimulant and the reinforcing/euphoric effects of abused drugs are mediated by the same neural circuit. However, this is not a widely accepted view. While acknowledging the mesolimbic dopamine system as an important substrate for these processes, Koob and collegues have argued that not all drugs of abuse produce their rewarding and stimulant effects by directly activating this system (Koob and Bloom, 1988; Koob, 1992; Koob et al., 1998). Koob and collegues suggest that EtOH's rewarding effects may also be mediated, in part, by neural structures that indirectly influence this circuit (Koob et al., 1998). Nevertheless, sensitivity to EtOH's locomotor

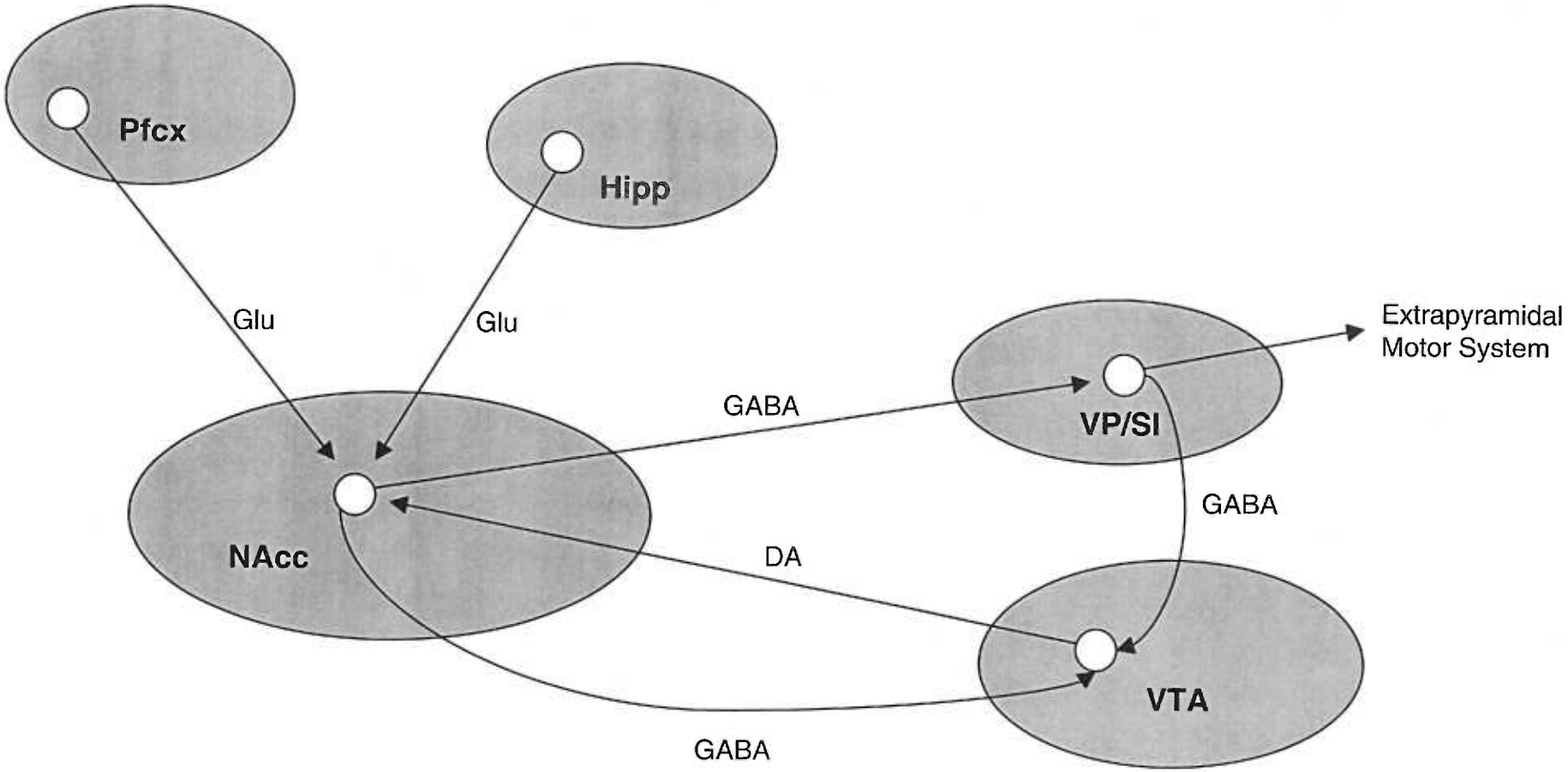
stimulant effects may be a relevant trait marker for sensitivity to the development of alcohol abuse or alcoholism.

Mesolimbic Dopamine System

A simplified schematic representation of the midbrain mesolimbic dopamine (DA) system can be seen in Figure 1. The principal components of this system are DA projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc). However, the VTA also sends DA projections to the olfactory tubercle, frontal cortex, and amygdala. VTA DA neurons projecting to the NAcc synapse on γ-aminobutyric acid (GABA) neurons, which then project to the ventral pallidum/substantia innominata (VP/SI) where they modulate output to the extrapyramidal motor system (Amalric and Koob, 1993; Hooks and Kalivas, 1995; Kalivas and Nakamura, 1999). As seen in Figure 1, there is also a glutamate (Glu) presence within the circuit. The NAcc receives Glu projections from the prefrontal cortex and hippocampus. Completing a negative feedback loop, the VTA receives GABA projections from the NAcc and the VP/SI (Pierce and Kalivas, 1997).

As do other abused drugs, EtOH activates the mesolimbic DA pathway. Using *in vivo* microdialysis in freely moving rats, Imperato and Di Chiara (1986) showed that low doses of EtOH increased the release of DA, as well as its metabolites 3,4-dihydroxyphenyl-acetic acid and homovanillic acid, in the NAcc. Importantly, these changes in DA release were accompanied by a locomotor stimulant response. Since

Figure 1. Schematic representation of mesolimbic dopamine (DA) projection. Ventral Tegmental Area (VTA) dopamine neurons projecting to the nucleus accumbens (NAcc) synapse on γ-aminobutyric acid (GABA) neurons, which then project to the ventral pallidum/substantia innominata (VP/SI) where they modulate output to the extrapyramidal motor system. The NAcc receives glutamate (Glu) projections from the prefrontal cortex (Pfcx) and hippocampus (Hipp). Completing a negative feedback loop, the VTA receives GABA projections from the NAcc and the ventral pallidum/substantia innominata.


Pfcx
Hipp
Glu
Glu
GABA
Extrapyramidal
Motor System
VP/SI
GABA
NAcc
DA
VTA
GABA

this initial study, several other groups have reported similar findings (Kalivas and Stewart, 1991; Smith and Weiss, 1999).

EtOH has also been shown to excite DA cell bodies in the VTA directly. When low doses of EtOH were administered intravenously to rats, VTA DA neurons projecting to the NAcc were shown to be highly sensitive to EtOH (Gessa et al., 1995). Importantly, DA neurons originating in the substantia nigra, projecting to the striatum (nigrostriatal motor pathway), exhibited a 5-fold lower EtOH sensitivity, suggesting preferential sensitivity of mesolimbic DA neurons to EtOH. Similar findings have also been reported using *in vitro* preparations in both rats (Brodie et al., 1990; Brodie et al., 1999) and mice (Brodie and Appel, 2000). Thus, similar to other abused drugs, it appears that EtOH preferentially activates VTA DA neurons projecting to the NAcc.

Genetic Predisposition Influences Rodent Sensitivity to EtOH-Stimulated Activity

A popular approach to investigating genetic influences in alcohol abuse research is the study of genetic animal models, commonly genetic mouse models. Mice require little space, are relatively cheap to maintain, have short gestational periods, large litters, and are easy to breed. Furthermore, there is a significant evolutionary conservation of genes between the human and mouse genomes (Copeland et al., 1993; Hudson et al, 2001). That is, most, if not all, mouse genes have homologs in the human genome. Thus, if a gene influencing sensitivity to EtOH's locomotor stimulant effects is isolated in the mouse, it is likely that a similar gene can be located in humans.

Studies using genetic mouse models have supported human data in suggesting that genetic predisposition influences sensitivity to the locomotor stimulant effects of

EtOH. These studies have shown pronounced differences in sensitivity to the locomotor stimulant effects of EtOH among inbred (Crabbe et al., 1982; Crabbe, 1986; Cunningham et al., 1992; Dudek and Tritto, 1994) and recombinant inbred mouse strains (Phillips et al., 1995). Moreover, our laboratory has selectively bred mice for differential sensitivity to EtOH's locomotor stimulant effects (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995; Phillips et al., *submitted*). The pattern of response over generations of selective breeding of these mouse lines, as well as quantitative trait locus (QTL) mapping data obtained from the BXD recombinant inbred mouse panel (Phillips et al., 1995) and other mapping populations (Demarest et al., 2001), suggest that sensitivity to the locomotor stimulant effects of EtOH is a polygenic trait (i.e., a number of genes influence sensitivity to this EtOH effect). In particular, QTL mapping has identified several chromosomal regions that each contains one or more genes influencing sensitivity to EtOH's locomotor stimulant effects, each locus making a small contribution to the total genetic influence on the trait (genotypic variance).

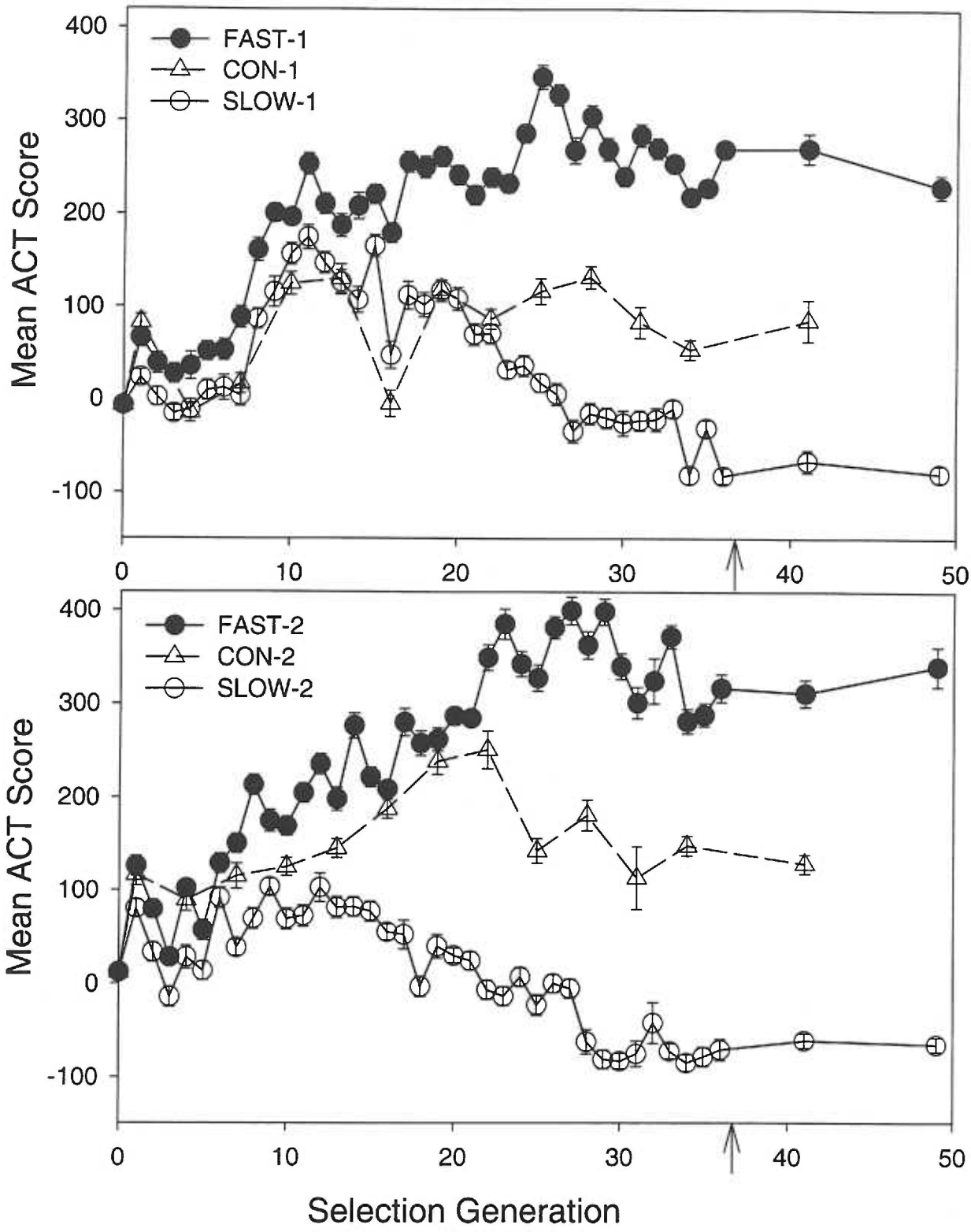
The FAST and SLOW Selected Mouse Lines

Selective breeding is a process by which the frequencies of alleles influencing a trait are systematically altered within a population. The FAST and SLOW mouse populations were selectively bred for differential sensitivity to EtOH-stimulated locomotor activity (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995; see Figure 2). Prior to selection generation 6, breeders for subsequent generations of FAST and SLOW mice were chosen based on the magnitude of difference in locomotor response to saline administration on one day, and 1.5 g/kg EtOH (20% v/v) injection the next day

(testing occurred under dim lighting conditions). However, from selection generation 6 on, breeders were chosen based on locomotor response to 2 g/kg EtOH (20% v/v), the order of EtOH and saline testing was reversed (EtOH on day 1, saline on day 2), and the mice were tested under bright ambient light (Phillips et al., 1991). These changes in the selection protocol were made due to the apparent lack of a robust response to selection early on in the project (see Figure 2), and were based on data suggesting that the order of drug administration (saline versus EtOH), the light intensity within the testing chambers, and the EtOH dose have profound effects on the magnitude of EtOH-stimulation in FAST and other mouse models (Middaugh et al., 1987; Lister, 1987; Crabbe et al., 1988).

Two separate populations of mice exhibiting extreme sensitivity to EtOH's locomotor stimulant effects were mated in a within-family breeding scheme to generate the FAST-1 and -2 selected lines, whereas two additional populations of resistant mice were similarly mated to generate the SLOW-1 and –2 selected lines. After 37 generations of selective breeding, FAST-1 and -2 mice exhibit extreme sensitivity, whereas SLOW-1 and -2 mice are insensitive, to EtOH's locomotor stimulant effects (Figure 2). The replicate lines were produced from independent populations to control for chance fixation of alleles that do not influence sensitivity to EtOH-stimulated activity; it is less likely, for instance, that the same allele would be fixed or increased in frequency in both the FAST-1 and -2 replicate lines unless the allele enhanced sensitivity to EtOH's locomotor stimulant effects. The FAST and SLOW selected mouse lines are useful animal models for elucidating the mechanisms mediating EtOH-stimulated locomotor activity. Furthermore, because of the relationship between EtOH's locomotor stimulant

Figure 2. Response to selection of the FAST-1 and SLOW-1 (upper panel), and FAST-2 and SLOW-2 (lower panel) selectively bred mouse lines through generation 36 (figure borrowed from Phillips et al., *submitted*). ACT scores represent the locomotor response to 2 g/kg EtOH (IP) on day 1, minus the locomotor response to saline vehicle on day 2, except for the first 5 generations, were 1.5 g/kg EtOH was administered and the order of testing was reversed (see Phillips et al., 1991). Photocell beam interruptions were counted during a 4 min test initiated 2 min after EtOH or saline injection. The means of two non-selected control lines (CON-1 and -2) are also shown. Selection was relaxed after 37 generations of selective breeding (indicated by the arrows), and mean ACT scores for first-litter offspring after four and twelve generations of relaxed selection are shown at 41 and 49 on the x-axis.


FAST-1
CON-1
SLOW-1
Mean ACT Score
400
300
200
100
0
-100
0
10
20
30
40
50
FAST-2
CON-2
SLOW-2
Mean ACT Score
400
300
200
100
0
-100
0
10
20
30
40
50
Selection Generation

and euphoric/rewarding effects, identifying the mechanisms that mediate EtOH-stimulated activity in FAST and SLOW mice my ultimately aid in our understanding of the mechanisms leading to EtOH addiction.

Neural Basis of EtOH's Effects in FAST and SLOW Mice

It is reasonable to suspect that EtOH produces its locomotor stimulant effects by interacting with a number of different neurotransmitter systems. The DA projection from the VTA to the NAcc has been postulated to be a primary mediator of EtOH-stimulated activity (Imperato and Di Chiara, 1986; Kalivas and Stewart, 1991; Smith and Weiss, 1999). However, as previously discussed, both glutamate and GABA modulate the activity of this projection (Amalric and Koob, 1993; Hooks and Kalivas, 1995; Pierce and Kalivas, 1997; Kalivas and Nakamura, 1999). Thus, it is logical to speculate that glutamate and GABA systems, and/or the DA system, may have been differentially altered during genetic selection of the FAST and SLOW mouse lines. Interestingly, pharmacological and/or neurochemical studies support a role for each of these neurotransmitter systems in mediating differences in sensitivity to EtOH's stimulant effects in these mice.

Dopamine. Consistant with its known involvement in mediating the locomotor stimulant response to a number of abused drugs including EtOH (Phillips and Shen, 1996), pharmacological evidence suggests a significant role for the DA system in the mediation of EtOH's locomotor stimulant effects in FAST mice (Shen et al., 1993). The DA D_1 receptor antagonist SCH-23390, and the D_2 receptor antagonist raclopride, dose-dependently attenuated locomotion in EtOH-naïve FAST and SLOW mice. However, the

lines were not differently sensitive to this effect. When administered prior to EtOH injection, DA receptor antagonists attenuated EtOH's locomotor stimulant effects; raclopride and haloperidol (mixed D_1 and D_2 DA receptor antagonist) dose-dependently attenuated these EtOH effects in both FAST-1 and -2 mice, whereas SCH-23390 dose-dependently attenuated these effects in FAST-1 mice only. Importantly, these effects occurred at doses that did not alter locomotion in the absence of EtOH. Furthermore, coadministration of SCH-23390 and raclopride decreased EtOH-stimulated locomotion to a greater extent then either drug alone. These drugs did not alter EtOH's effects on activity in SLOW mice. However, selection for reduced sensitivity to EtOH stimulation has resulted in the complete absence of a stimulated response in the two SLOW lines.

Studies investigating the effects of DA receptor agonists also support a role for DA receptors in mediating EtOH's effects in FAST mice (Phillips and Shen, 1996). Administration of SKF 38393 (partial D_1 receptor agonist) or quinpirole (D2/D3 receptor agonist) significantly enhanced EtOH's locomotor stimulant effects in FAST-1 mice, without having any effect on general locomotion. In contrast, these drugs did not alter EtOH's stimulant effects in FAST-2 mice, possibly because EtOH had maximally stimulated locomotion, rendering SKF 38393 incapable of any further enhancement. This contention is based on the relatively greater EtOH stimulant response seen in FAST-2 relative to FAST-1 mice. SKF 38393 did not alter basal locomotion in FAST-2 mice. Thus, experiments examining the effects of DA receptor agonists, as well as antagonists, support a role for DA in the mediation of EtOH's locomotor stimulant effects in FAST mice. The effects of DA receptor agonists were not tested in SLOW mice.

Published studies examining the effects of addictive drugs known to enhance DA availability at its receptors did not support a role for the DA system in the mediating the differential sensitivity to EtOH's stimulant effects in FAST and SLOW mice. However, more recent, unpublished data have changed that picture. The function of the DA transporter is reversed by d-amphetamine, resulting in increased DA in the synaptic cleft. This drug was administered to FAST and SLOW mice in two different experiments. In one study, d-amphetamine (2.5-10 mg/kg) dose-dependently increased locomotor activity in mice ($S_{11}G_{11}$), but the lines did not differ in sensitivity to this effect (Phillips et al., 1992). NOTE: S_x refers to selection generation, G_x refers to overall number of generations. However, in a recent unpublished study, similar doses of d-amphetamine dose-dependently stimulated locomotion in FAST-1 compared to SLOW-1 mice (FAST-1 > SLOW-1), but FAST-2 and SLOW-2 mice ($S_{37}G_{40}$) exhibited similar stimulant responses. Cocaine blocks the DA transporter, resulting in prolonged availability of DA in the synaptic cleft. Cocaine (5-40 mg/kg) dose-dependently stimulated locomotor activity to a similar extent in $S_{11}G_{11}$ FAST and SLOW mice (Phillips and Shen, 1996). However, similar to the story for d-amphetamine, a recent unpublished study found that whereas $S_{37}G_{40}$ FAST-2 and SLOW-2 mice did not differ in sensitivity to the locomotor stimulant effects of cocaine, FAST-1 mice exhibited a greater sensitivity than SLOW-1 mice. Taken together, the results of these experiments suggest that continued selection pressure differentially altered genes that influence sensitivity to d-amphetamine and cocaine in the replicate 1 FAST and SLOW lines, but not the replicate 2 lines. The likelihood that differential sensitivity to d-amphetamine and cocaine is genetically

correlated with EtOH sensitivity (i.e., the responses involve common genes) is currently being investigated.

Glutamate. In addition to investigations into DA's involvement in EtOH-stimulated activity in FAST and SLOW mice, some data suggest the involvement of Glu, the major excitatory neurotransmitter in the brain, in mediating the effects of EtOH. In particular, EtOH has been shown to inhibit the function of the N-methyl-D-aspartate (NMDA) Glu receptor. Activation of these voltage-gated receptors results in the influx of Ca^{2+}, Na^{+}, and K^{+} ions into the cell, resulting in neuronal depolarization, and EtOH has been shown to decrease NMDA-stimulated increases in intracellular free Ca^{2+} (Dildy-Mayfield and Leslie, 1991). Interestingly, both the VTA (from the prefrontal cortex) and the NAcc (from the prefrontal cortex and hippocampus) receive Glu inputs (Amalric and Koob, 1993; Hooks and Kalivas, 1995; Pierce and Kalivas, 1997; Kalivas and Nakamura, 1999), and the noncompetitive receptor antagonist, MK-801, has been shown to alter EtOH's locomotor stimulant effects (Liljequist, 1991; Robledo et al., 1991; Criswell et al., 1994; Kuribara, 1994). However, these studies disagree on the direction of this modulatory action, possibly due to the different doses of MK-801 tested.

Two studies have suggested a role for the Glu system in mediating EtOH's locomotor stimulant effects in FAST and SLOW mice. In one study, L-glutamate-stimulated increases in intracellular free Ca^{2+} concentration were determined in hippocampal and cortical microsacs of FAST and SLOW mice (Daniell and Phillips, 1994). This cell-free brain membrane preparation has been used to assess NMDA receptor function. The lines did not differ in basal L-glutamate-stimulated increases in intracellular free Ca^{2+}. However, compared to that of FAST-1 and -2 mice, EtOH

produced a significant concentration-dependent decrease in L-glutamate-stimulated intracellular free Ca^{2+} in the hippocampal and cortical microsacs isolated from SLOW-1 and -2 mice. These results suggest that the NMDA receptors of SLOW mice are more sensitive to the inhibitory effects of EtOH than are the NMDA receptors of FAST mice.

Behavioral data in FAST and SLOW mice also suggest a role for the Glu system in the mediation of EtOH's locomotor stimulant effects (Shen and Phillips, 1998). When MK-801 (0.01-0.5 mg/kg) was administered in the absence of EtOH, it had biphasic effects in both FAST and SLOW mice, stimulating locomotion at lower doses, and suppressing it at higher doses. Interestingly, FAST mice were more sensitive to both effects than were SLOW mice. When MK-801 was administered just prior to EtOH injection, doses that stimulated activity on their own significantly attenuated EtOH-stimulated activity in FAST-1 and -2 mice, and potentiated the locomotor depressant effects of EtOH in SLOW-1 and -2 mice. Thus, NMDA receptor mediated systems also appear to play a role in EtOH's locomotor stimulant effects in FAST and SLOW mice. Other glutamatergic drugs are currently under investigation.

$GABA_A$ receptors. The GABA system, the major inhibitory neurotransmitter system in the brain, has also been implicated in mediating EtOH's locomotor stimulant effects. Indeed, the NAcc sends GABA projections to the VP/SI, and the VTA receives GABA inputs from the NAcc and VP/SI (Amalric and Koob, 1993; Hooks and Kalivas, 1995; Kalivas and Nakamura, 1999). Thus, GABA has a significant presence within the mesolimbic DA system. Two major classes of GABA receptors have been described, the $GABA_A$ and $GABA_B$ receptor classes.

The $GABA_A$ receptor is a ligand-gated ion-channel. When GABA binds the receptor-channel complex, Cl^- ions are allowed to pass through the channel, and into the cell, resulting in a rapid neuronal hyperpolarization. EtOH is thought to produce a number of its effects by allosterically modulating the function of the $GABA_A$ receptor-channel (Deitrich et al., 1989; Ueno et al., 2001), and site-directed mutagenesis has identified a putative $GABA_A$ receptor binding-site for the drug (Mascia et al., 2000). The $GABA_A$ receptor also possess several other binding sites. Barbiturates, benzodiazepines, and neuroactive steroids each bind at a separate site on the $GABA_A$ receptor and allosterically modulate Cl^- flux through the channel.

An early report suggested that the FAST and SLOW selected mouse lines differ in sensitivity to several $GABA_A$ receptor ligands. Phillips et al. (1992) demonstrated differential sensitivity of $S_{13\text{-}21}G_{13\text{-}21}$ FAST and SLOW mice to the locomotor stimulant effects of several drugs that allosterically modulate Cl^- flux through the receptor channel; FAST-1 and -2 mice were more sensitive to the locomotor stimulant effects of alcohols (methanol, t-butanol, and n-propanol) and barbiturates (pentobarbital and phenobarbital). Moreover, FAST-1 and -2 mice were less sensitive to the locomotor sedative effects of the benzodiazepine, diazepam. More recently, these findings were extended when Shen et al. (1998) investigated the effects of diazepam, and another benzodiazepine, midazolam, in FAST and SLOW mice from $S_{22\text{-}28}G_{22\text{-}38}$. Diazepam had similar effects to those reported in the earlier study, with FAST-1 and -2 mice exhibiting relative resistance to its locomotor sedative effects. FAST-1 and –2 mice were more sensitive to midazolam's locomotor stimulant properties than were SLOW-1 and –2 mice. Finally, a recent study investigated the effects of allopregnanolone, a neuroactive steroid, in FAST

and SLOW mice (Palmer et al., *in press*). Similar to other allosteric modulators of the $GABA_A$ receptor channel, allopregnanolone binds at a separate and distinct site from the GABA binding site. Lower doses of allopregnanolone dose-dependently stimulated locomotion in FAST-1 and -2 mice, but had little effect in SLOW-1 and -2 mice. Allopregnanolone produced a locomotor sedative effect in all lines at the higher doses tested.

The effects of drugs that competitively bind the GABA site of the $GABA_A$ receptor have also been investigated in FAST and SLOW mice (Shen et al., 1998). Administration of the $GABA_A$ receptor agonist, muscimol, dose-dependently attenuated general locomotion in both FAST and SLOW mice, however the lines did not differ in sensitivity to this effect. Similar effects were seen when bicuculline, a $GABA_A$ receptor antagonist, was tested in FAST and SLOW mice. That both the agonist and antagonist would attenuate general locomotion is puzzling but could be related to the range of doses examined. However, bicuculline also failed to alter EtOH's locomotor stimulant effects in FAST mice. Taken together, these data suggest that the GABA binding site of the $GABA_A$ receptor is not a likely candidate for $GABA_A$ receptor alterations that may have accompanied selection for differential sensitivity to EtOH-stimulated locomotion. Considered along with the effects of barbiturate, benzodiazepine, and neuroactive steroid administration, these data suggest that genetic selection for differential sensitivity to EtOH's locomotor stimulant effects altered sensitivity to allosteric modulators of the $GABA_A$ receptor, and not to drugs that directly bind the GABA site. Of final note, picrotoxin, a non-competitive $GABA_A$ receptor antagonist that blocks the Cl^- channel,

Table 1. Summary of results from pharmacological studies investigating neurotransmitter receptors involved in EtOH's locomotor effects in FAST and SLOW mice.

DRUG	Receptor Interaction	Drug Effect	Relative Sensitivity to Drug	Effect of Drug on EtOH-altered Locomotion	Citation
Dopaminergic					
SCH-23390	D_1 antagonist	Locomotor Depression	FAST-1=SLOW-1 FAST-2=SLOW-2	↓ stim in FAST-1 and FAST-2 no change in SLOW	Shen et al., 1993
Raclopride	D_2 antagonist	Locomotor Depression	FAST-1=SLOW-1 FAST-2=SLOW-2	↓ stim in FAST-1 and FAST-2 no change in SLOW	Shen et al., 1993
Haloperidol	D_1/D_2 antagonist	Not examined	Not examined	↓ stim in FAST-1 and FAST-2 no change in SLOW	Shen et al., 1993
SKF 38393	Partial D_1 agonist	Not examined	Not examined	↑ stim in FAST-1 no effect in FAST-2 SLOW not tested	Phillips and Shen, 1996
Quinpirole	D_2/D_3 agonist	Not examined	Not examined	↑ stim in FAST-1 no effect FAST-2 SLOW not tested	Phillips and Shen, 1996
d-Amphetamine	Transporter blocker	Dose-dependent stimulation	FAST-1›SLOW-1 FAST-2=SLOW-2	Not tested	Phillips et al., unpublished
Cocaine	Transporter blocker	Dose-dependent stimulation	FAST-1›SLOW-1 FAST-2=SLOW-2	Not tested	Phillips et al., unpublished
Glutamatergic					
MK-801	NMDA antagonist	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	↓ stim in FAST-1 and FAST-2 ↑ depr in SLOW-1 and SLOW-2	Shen and Phillips, 1998
GABAergic					
Muscimol	$GABA_A$ agonist	Locomotor depression	FAST-1=SLOW-1 FAST-2=SLOW-2	Not tested	Shen et al., 1998
Bicuculline	$GABA_A$ antagonist	Locomotor depression	FAST-1=SLOW-1 FAST-2=SLOW-2	Not tested	Shen et al., 1998
Methanol	$GABA_A$ modulator	Biphasic*	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Phillips et al., 1992
t-Butanol	$GABA_A$ modulator	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Phillips et al., 1992
n-Propanol	$GABA_A$ modulator	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Phillips et al., 1992
Pentobarbital	$GABA_A$ modulator	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Phillips et al., 1992
Phenobarbital	$GABA_A$ modulator	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Phillips et al., 1992
Diazepam	$GABA_A$ modulator	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Phillips et al., 1992; Shen et al., 1998
Midazolam	$GABA_A$ modulator	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Shen et al., 1998
Allopreganolone	$GABA_A$ modulator	Biphasic	FAST-1›SLOW-1 FAST-2›SLOW-2	Not tested	Palmer et al, *in press*
Baclofen	$GABA_B$ agonist	Locomotor depression	FAST-1‹SLOW-1 FAST-2=SLOW-2	↓ stim in FAST-1 and FAST-2 SLOW not tested	Shen et al., 1998

*Biphasic: Locomotor stimulation at lower doses, and locomotor depression at higher doses. Relative sensitivity for these drugs refers specifically to stimulant doses; stim: locomotor stimulation; depr: locomotor depression. See text pages 14-26 for a full description of the data summarized in this table. The symbols, <,>, and = indicate relative sensitivity.

also dose-dependently reduced general locomotion in FAST and SLOW mice, but the lines did not differ in sensitivity to this effect.

$GABA_B$ receptors. The above review of the FAST and SLOW literature is summarized in Table 1. Studies investigating DA and GABA involvement in mediating the differential effects of EtOH on locomotion in FAST and SLOW mice, make clear that differential sensitivity is consistent for drugs that allosterically modulate the $GABA_A$ receptor/channel. However, whereas the $GABA_A$ system has been extensively investigated, the $GABA_B$ system has not been as well studied. Interestingly, as will be seen in subsequent sections, $GABA_B$ receptor ligands have been implicated in mediating EtOH's locomotor stimulant effects in various mouse models (Cott et al., 1976; Allan and Harris, 1989; Hemuniuk et al., 1993). $GABA_B$ receptors are known to reside on DA cell bodies in the VTA where they could modulate EtOH-stimulated DA release (Grace and Bunney, 1984; Lacey et al., 1988), and possibly EtOH-stimulated locomotion. Thus, the $GABA_B$ receptor system would appear to be a likely mediator of sensitivity to EtOH's locomotor stimulant effects in FAST and SLOW mice. Interestingly, a $GABA_B$ receptor agonist dose-dependently attenuated EtOH-stimulated locomotor activity in FAST mice (Shen et al., 1998). Furthermore, there is some evidence from the same study to suggest that the lines may differ in $GABA_B$ receptor agonist sensitivity in the absence of EtOH.

Based on these very interesting findings, and the fact that examination of the role of $GABA_B$ receptors has been less extensive in this model of extreme sensitivity and insensitivity to EtOH, I chose to focus on this neurotransmitter receptor for further investigation in my dissertation project. Thus, a more thorough review of the literature

pertaining to this neurotransmitter system, and its relationship to EtOH-stimulated locomotor activity follows.

$GABA_B$ Receptor Modulation of the Locomotor Stimulant Effects of EtOH

EtOH administration increases DA release in the NAcc to a greater extent in rats that exhibit a locomotor stimulant response than in those that do not (Smith and Weiss, 1999). Presumably, a similar effect would be seen if microdialysis were performed in mice. Postsynaptic $GABA_B$ receptors localized on DA cell bodies in the VTA may modulate these EtOH effects. $GABA_B$ receptors are inhibitory G-protein (*Gi*) coupled receptors that, when bound by agonist, inhibit adenylate cyclase (Andrade et al., 1986; Hill, 1985; Karbon and Enna, 1985; Wojcik and Neff, 1984). Research suggests that presynaptic $GABA_B$ receptor activation reduces Ca^{2+} conductance leading to a decrease in neurotransmitter release (Cherubini and North, 1984; Deisz and Lux, 1985) and that postsynaptic $GABA_B$ receptor activation increases K^+ conductance resulting in neuronal hyperpolarization (Inoue et al., 1985a; Inoue et al., 1985b). Thus, postsynaptic $GABA_B$ receptors located in the VTA may reduce DA release in the NAcc by hyperpolarizing the DA cell bodies in the VTA (Grace and Bunney, 1984; Lacey et al., 1988).

Pharmacological studies support a role for $GABA_B$ receptor modulation of EtOH-stimulated locomotor activity. In the earliest report, Cott et al. (1976) showed that administration of the $GABA_B$ receptor agonist, baclofen attenuated the locomotor stimulant effects of EtOH in NMRI mice. Similar results were seen when phaclofen, a $GABA_B$ receptor antagonist, was administered to Swiss-Webster mice (Allan and Harris,

1989). That an agonist and antagonist of the $GABA_B$ receptor would have the same effect on EtOH-stimulated behavior was at first perplexing. However, in a more recent study using BALB/c inbred mice, Humeniuk et al. (1993) provided additional evidence that baclofen attenuates EtOH's locomotor stimulant effects. These authors showed attenuation of EtOH stimulation at baclofen doses that did not alter locomotor activity in the absence of EtOH. This is important since baclofen was known to have locomotor sedative effects at higher doses in rats (Parades and Agmo, 1989). Hemuniuk et al. acknowledged the data presented by Allan and Harris (1989), showing that phaclofen had effects on EtOH-stimulated activity similar to those of baclofen. However, these authors argued that phaclofen is a very weak $GABA_B$ receptor antagonist, and that in the study by Allan and Harris (1989), this drug was likely acting as an agonist, thus having effects similar to baclofen.

Evidence suggests that selection for differential sensitivity to EtOH's locomotor stimulant effects differentially altered the $GABA_B$ receptor system in FAST and SLOW mice. In a recent report by Shen et al. (1998), baclofen produced a dose-dependent locomotor sedative effect in EtOH-naïve FAST and SLOW mice. FAST-2 and SLOW-2 mice did not differ in sensitivity to this baclofen effect at the range of doses tested. However, FAST-1 mice exhibited reduced sensitivity to baclofen compared to SLOW-1 mice, providing moderate evidence that genetic selection might have resulted in increased $GABA_B$ receptor density or enhanced $GABA_B$ receptor function in SLOW-1, relative to FAST-1 mice. Selection could also have altered some other neural system influenced by the $GABA_B$ receptor system. When baclofen was administered prior to EtOH injection, it attenuated the locomotor stimulant effects of EtOH in FAST mice at doses that did not

alter general locomotion, and this effect was reversed by administration of the $GABA_B$ receptor antagonist CGP-35348 (Shen et al., 1998). Because SLOW mice do not exhibit a locomotor response to EtOH, baclofen's effects in EtOH-treated SLOW mice were not investigated. However, in the same study, similar results were produced when baclofen was administered to EtOH-treated DBA/2J inbred mice, a mouse strain that is sensitive to the stimulant effects of EtOH; baclofen dose-dependently attenuated EtOH-stimulated activity, and this effect was reversed by CGP-35348 (Shen et al., 1998).

Specific Aims

Aim 1. In the previous section, data were reviewed showing that baclofen dose-dependently attenuated the locomotor stimulant effects of EtOH in FAST mice (Shen et al., 1998). Similar results were obtained when baclofen was administered via intracerebroventricular (ICV) injection. These results are reported in Chapter 1 of this dissertation only because that chapter was written in publication format, and they were included in the manuscript we recently submitted for publication (Boehm II et al., *submitted*). The experiment was designed by Dr. T. Phillips and Marla Piercy, and executed by Marla Piercy. Marla Piercy was a former graduate student in the laboratory. The results of this ICV injection experiment suggest that baclofen can alter EtOH-stimulated locomotion via a central site of action, an important finding because $GABA_B$ receptors are known to reside in the spinal cord where they mediate baclofen's antispastic effects (Taricco et al., 2000). These results do not specify the central site of baclofen's effects, but a likely target is the VTA. Given that increased DA release is associated with

EtOH's locomotor stimulant effects, $GABA_B$ receptors in the VTA may negatively modulate EtOH-stimulated locomotor activity. Investigation of this hypothesis was the first aim of this dissertation project.

For the experiments reported in Chapter 1, FAST-1 and -2 mice received bilateral intra-VTA microinjections of baclofen, were then injected systemically with EtOH or saline, and immediately tested in automated locomotor activity monitors for 30 min. We hypothesized that intra-VTA baclofen would dose-dependently attenuate EtOH's locomotor stimulant effects in FAST mice, having little effect in the absence of EtOH. However, preliminary data suggested that the effects of intra-VTA baclofen depended on whether baclofen was microinjected into the anterior or posterior region of the VTA in FAST mice. Thus, based on these preliminary findings, intra-VTA microinjections of baclofen were aimed at both the anterior and posterior regions of the VTA. Furthermore, SLOW-1 and -2 mice were included in the experiment after the preliminary study suggested that more posterior intra-VTA injections of baclofen potentiate EtOH's locomotor stimulant effects in FAST mice. We speculated that posterior intra-VTA microinjection of baclofen might induce EtOH-stimulated locomotion in SLOW mice.

Aim 2. $GABA_B$ receptors and their transcripts have been localized in many brain regions including the VTA, cortex, basal forebrain, hippocampus, thalamus, hypothalamus, cerebellum, and midbrain in rats (Kaupmann et al., 1997; Chu et al., 1990; Bowery et al., 1987). Thus, it is possible that the effects of intra-VTA baclofen could be mediated by neural structures very near the VTA. In particular, the substantia nigra pars compacta (SNC), a midbrain structure located ventrolateral to the VTA and thought to control general locomotion, possesses an abundance of $GABA_B$ receptors (Kaupmann et

al., 1997; Chu et al., 1990; Bowery et al., 1987). Aim 2 first sought to determine whether FAST and SLOW mice differ in density of $GABA_B$ receptors in the anterior and posterior region of the VTA to examine the hypothesis that changes in the number of these receptors accompanied selection for differential sensitivity. In addition, $GABA_B$ receptor density was examined in the nearby SNC, due to its proximity to the microinjection site and the possibility that receptor density differences could be associated with the different baclofen effects in FAST and SLOW mice.

The brains of FAST-1 and -2 and SLOW-1 and -2 mice were cut into 16 μm thick sections and subjected to $GABA_B$ receptor autoradiographic analysis of the VTA and SNC using [^{3}H]CGP 54626, a $GABA_B$ receptor antagonist. SLOW-1 mice were more sensitive than FAST-1 mice to the locomotor sedative effects of systemic baclofen (Shen et al., 1998). Thus, we hypothesized that compared to FAST mice, SLOW mice would possess a greater density of $GABA_B$ receptors in the VTA. We also hypothesized that the lines would not differ in [^{3}H]CGP-54626 binding in the SNC. If a significant line difference in $GABA_B$ receptor density was found in the VTA but not in the SNC, these results would provide supportive evidence that intra-VTA baclofen alters locomotion in the FAST and SLOW lines at the level of the VTA, rather than by diffusing to the SNC.

CHAPTER 1

Ventral Tegmental Area Region Governs $GABA_B$ Receptor Modulation of Ethanol-Stimulated Activity in Mice.

Introduction

Identification of neurochemical mechanisms mediating genetic susceptibility to traits associated with alcoholism is key to treatment and prevention. However, heritable risk factors have been difficult to identify. Relative sensitivity to alcohol's depressant effects (Schuckit and Smith, 2000; 2001; Heath et al., 2001), and arousing, or stimulant effects (Newlin and Thomson, 1999; Poikolainen, 2000; Holdstock et al., 2000) appear to be such behavioral traits. Studies in mice have demonstrated that sensitivity to the locomotor stimulant effects of alcohol (ethanol; EtOH) is heritable (Crabbe et al., 1987; Dudek et al., 1990; Crabbe et al., 1994; Shen et al., 1995). Moreover, similar neurochemical changes have been associated with EtOH's locomotor stimulant effects and its rewarding properties (Kalivas et al., 1990; Amalric and Koob, 1993; Hooks and Kalivas, 1995). Thus, further investigation of neurochemical mechanisms underlying EtOH's locomotor stimulant effects in mice may be relevant to understanding EtOH's addictive properties.

The locomotor stimulant effects of EtOH, and other abused drugs, appear to be mediated by the mesoaccumbens-pallidal circuit (Amalric and Koob, 1993; Hooks and Kalivas, 1995; Kalivas and Nakamura, 1999). This circuit comprises dopamine (DA) neurons in the ventral tegmental area (VTA) that project to gamma-aminobutyric acid

(GABA) neurons in the nucleus accumbens (NAcc). These GABA neurons project to the ventral pallidum/substantia innominata where they modulate output to the extrapyramidal system. Abused drugs such as cocaine, amphetamine, and EtOH increase DA release in the NAcc (Kalivas and Stewart, 1991; Smith and Weiss, 1999).

$GABA_B$ receptors are G-protein (G*i*) coupled receptors that inhibit adenylate cyclase (Wojcik and Neff, 1984; Hill, 1985; Karbon and Enna, 1985; Andrade et al., 1986). Presynaptic $GABA_B$ receptor activation reduces Ca^{2+} conductance, decreasing neurotransmitter release (Cherubini and North, 1984; Deisz and Lux, 1985), whereas postsynaptic $GABA_B$ receptor activation increases K^+ conductance, resulting in neuronal hyperpolarization (Inoue et al., 1985a; Inoue et al., 1985b). Thus, postsynaptic $GABA_B$ receptors, known to reside on DA cell bodies in the VTA, may reduce DA release in the NAcc by hyperpolarizing VTA DA cell bodies (Grace and Bunney, 1984; Lacey et al., 1988).

Pharmacological studies support a role for $GABA_B$ receptors in modulating EtOH's stimulant effects (Cott et al., 1976; Allan and Harris, 1989; Humeniuk et al., 1993). Our laboratory has shown that systemic injection of baclofen, a $GABA_B$ agonist, attenuates the locomotor stimulant effects of EtOH in FAST mice, selectively bred for extreme sensitivity to this effect (Shen et al., 1998).

$GABA_B$ receptors in the VTA may modulate sensitivity to EtOH's stimulant effects in FAST mice by negatively modulating associated DA release in the NAcc. However, $GABA_B$ receptors are also found in the spinal cord (Towers et al., 2000). Indeed, baclofen is a widely prescribed muscle relaxant (see review by Taricco et al., 2000). Thus, baclofen could conceivably reduce EtOH's locomotor stimulant effects via

actions outside the brain. Here we first sought to identify a central role for baclofen's effects on EtOH-stimulated activity by performing intracerebroventricular (ICV) injections of the drug. Next, we investigated the VTA as a potential mediator of these effects by microinjecting baclofen directly into the structure.

Methods

Animals.

Originating from the genetically heterogeneous HS/ibg mouse stock, FAST-1 and -2, and SLOW-1 and -2 mice, were independently produced by selective breeding for high and low sensitivity to EtOH-induced locomotor stimulation, respectively (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995). Selection was terminated after 37 generations, and these lines are now maintained at the Portland VA Medical Center under relaxed selection. Selection was based on locomotor response to 2.0 g/kg EtOH (20% v/v) on one day of behavioral testing, corrected by subtraction of locomotor activity after saline treatment the next day. Photocell beam interruptions were recorded in Lehigh Valley circular open-field activity monitors, and the EtOH and saline activity tests occurred at 24 hr intervals. The highest scoring male and female from each of 9 families per replicate line was mated in a rotational within-family breeding scheme to produce FAST-1 and -2 offspring for the next generation. Likewise, those mice exhibiting extremely low scores were mated to produce SLOW-1 and -2 offspring. Brother-sister mating was prohibited. Mice for the current studies were from second or later litters of the primary FAST and SLOW breeders. They were housed with dam and sire until weaning at 21±2 days of age at which time they were housed 3-4 per cage in isosexual

groups with mice of the same genotype. Clear polycarbonate cages (28 by 18 by 13 cm) with corn-cob bedding were used, and mice had free access to standard rodent chow and water. All mice were maintained on a 12-hr light-dark cycle (lights on at 0600 hours), an ambient room temperature of 22 ±2°C, and were naïve at the time of testing. All procedures were approved by the Portland Veterans Affairs Medical Center Institutional Animal Care and Use Committee, and conformed with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals.*

Experiment 1. ICV injections of baclofen.

Subjects. Male and female FAST-1 and -2 mice (n's = 9-13 per replicate, BACLOFEN dose, and EtOH treatment) were used for the ICV experiments. It was not possible to obtain enough mice of one gender to generate adequate group sizes for this behavioral analysis. Thus, approximately equal numbers of mice of each gender were distributed across the groups. Mice were 50-90 days of age at the time of behavioral testing, and testing occurred between 0800 and 1600 hours.

ICV injections and behavioral testing. [Thesis Note: Marla Piercy, a former graduate student in the laboratory, performed all ICV injections and behavioral testing. This study is presented here only because it was included in the submitted manuscript. This experiment was not part of my thesis project.] The ICV injection technique was adapted from that of Laursen and Belknap (1986). Prior to ICV injections and behavioral testing, mice were moved to the locomotor testing room, weighed, and allowed at least 45 min to habituate to the testing room environment. ICV injections were performed under light isoflurane- (Burns Veterinary Supply, Rockville center, NY) induced anesthesia, chosen for its rapid induction, and short duration of action. Anesthesia was achieved by

individually placing mice in an OHMEDA (West Yorkshire, England) single induction oxygen-gas inhalation chamber (28x18x13 cm), equipped with a non-rebreathing circuit. An adequate level of anesthesia was achieved when shallow breathing was observed, usually within 2 min. A pilot study showed that the effects of isoflurane-induced anesthesia on basal locomotor behavior dissipated within 5 min of removal from the inhalation chamber.

Once an appropriate level of anesthesia was reached, mice were removed from the inhalation chamber, and immediately injected ICV with either artificial cerebrospinal fluid (*a*CSF; 140 mM NaCl, 3.36 mM KCL, 1.50 mM $CaCl_2$, 1.00 mM $MgCl_2$, 1.45 mM NaH_2PO_4, and 5.38 mM glucose, at pH 7.4) or one of three doses of baclofen. Pilot studies led to the choice of doses expected to have little, moderate, and maximal effects on EtOH-stimulated locomotion. Rather than injecting a constant concentration, concentrations were adjusted for male and female mice according to expected average body weight from pilot data. Body weight estimation was imperfect, but resulted in doses of baclofen that were approximately equivalent for male and female mice. Final doses were 0.72, 1.44, and 2.88 μg/kg for males, and 0.66, 1.33, and 2.66 μg/kg for females. In order to control the depth of the ICV injection, silastic tubing was slipped over a 25.4 mm, 27-gauge needle, 2.0 mm from the end of the bevel. The needle was attached to a 10 μl Hamilton glass syringe and inserted through the skull into the lateral ventricle (target coordinates with respect to bregma: 0.02 mm posterior, 1.0 mm medial, and 3.0 mm ventral). An injection volume of 2.0 μl was delivered. Upon completion of the ICV injection, mice were placed in holding cages for a 15-min recovery period. This recovery period was chosen to allow for adequate drug absorption, and because pilot data

showed a slight locomotor depressant response to ICV injection of *a*CSF that had completely dissipated 15 min after injection. Following recovery, mice were injected intraperitoneally (IP) with either EtOH (Pharmco Products, Brookfield, CT; 2.0 g/kg, 20% v/v in 0.09% saline) or an equivalent volume of saline, and immediately placed in a sound-attenuated automated activity monitor for locomotor activity data collection (Accuscan, Columbus, OH). This equipment was interfaced with an IBM-compatible computer, and testing continued for 60 min. Data were collected in 5-min time intervals.

Blood EtOH concentration. At the conclusion of behavioral testing, peri-orbital sinus blood (20 μl) was sampled from EtOH-treated mice for determination of blood EtOH concentration (BEC). These data were intended to assess possible effects of baclofen treatment on EtOH pharmacokinetics. Each blood sample was immediately placed in a microcentrifuge tube containing 50 μl of ice-cold $ZnSO_4$ and further processed as previously described (Boehm II et al., 2000). BECs were determined using a gas chromatograph (Hewlett-Packard 5890) with flame ionization detection.

Histological verification of injection sites. To verify injection sites, mice were deeply anesthetized (0.2 ml cocktail containing 5% ketamine, 2% xylazine, and 1% acepromazine; i.p.) and transcardially perfused with approximately 40 ml of fixative (5% glutaraldehyde, 0.6% paraformaldehyde, 0.1% picric acid, and 10% 0.1 M Hepes, at pH 7.3, and 84.3% Milli-Q filtered H_2O) at a flow rate of 20 ml/min using a Multiperpex peristaltic perfusion pump (H.J. Guldener, Zurich, Switzerland). The brains were removed, placed in fixative and chilled overnight (4 °C). Coronal brain slices (150 μm) were cut using a Pelco vibrotome (Technical Products Intl., St. Louis, MO). Slices were mounted on gelatin-coated slides and verified for injection placement. Data from animals

in which the needle tip penetrated only one lateral ventricle wall, and in which breakage of the interior epithelium wall of the lateral ventricle was observed, were included in the analysis. Animals that failed to meet these criteria were excluded from the study.

Statistical analysis. Locomotor activity and BEC data were analyzed by analysis of variance (ANOVA). Follow-up simple main effects analyses of 2-way interactions, and Neuman Keul's *post-hoc* comparisons were performed when necessary. The level of significance was set at $p < 0.05$.

Experiment 2. Intra-VTA microinjections of baclofen.

Subjects. Male FAST-1, FAST-2, SLOW-1, and SLOW-2 mice (n's = 4-10 per line, replicate, balcofen dose, EtOH treatment, and intra-VTA injection site) were used for intra-VTA microinjection experiments. Male mice were chosen based on no evidence of sex differences in response to ICV baclofen in Experiment 1. Following surgery, cannulated mice were housed in standard rat cages (30.8 by 30.8 by 19 cm, clear polycarbonate) with corn-cob bedding. Mice were 70-100 days of age at the beginning of behavioral testing. All behavioral testing occurred between 1200 and 1600 hours.

Bilateral cannulation of the VTA. Prior to stereotaxic surgery, mice were anesthetized using an anesthetic cocktail [acepromazine (1 ml of a 10 mg/ml stock solution), ketamine (5 ml of a 100 mg/ml solution), 0.9% saline (1.5 ml), and xylazine (2.5 ml of a 100 mg/ml stock solution)], diluted in 0.9% saline (1:6), administered IP in a volume of 10 ml/kg minus 0.125 ml. After an adequate level of anesthesia was achieved (assessed by the absence of a hind-foot withdrawal reflex following a light pinch), a small area of the dorsal scalp was shaved and a midline incision made, extending a few

millimeters anterior of bregma to a few millimeters posterior of lambda (about 3 mm wide). The mouse was then placed in a Cartesian Research Inc. (Sandy, OR) stereotaxic apparatus. A Styrofoam platform was constructed which maintained a flat position for the mouse and elevated its body to the level of the head holder. The exposed cranial surface was cleaned/sterilized with a 100% EtOH-soaked cotton swab, and the eyes were moistened with 0.9% saline.

Following these preparations, the distance between bregma and lambda was measured. This distance was used to modify the set of stereotaxic coordinates, taking the size of the skull/brain into account. The distance between bregma and lambda was divided by 3.39 mm, the published distance between bregma and lambda in the mouse brain atlas of Franklin and Paxinos (1997). Each predetermined coordinate was multiplied by the resultant value to obtain an adjusted set of stereotaxic coordinates (see below).

Two holes were drilled through the cranium for simultaneous placement of the guide cannulae. Early results suggested that the effects of intra-VTA baclofen on EtOH-stimulated activity depended on whether it was microinjected into the anterior or posterior VTA. Thus, our stereotaxic coordinates for the anterior VTA were 0.5 mm lateral, 2.5 mm posterior, and 2.0 mm ventral, with respect to bregma (Franklin and Paxinos, 1997). The coordinates for the posterior VTA were 0.5 mm lateral, 2.8 mm posterior, and 2.0 mm ventral, with respect to bregma. Once both holes were drilled, two guide cannulae (with stylets inserted; see below) were simultaneously lowered into the brain to the appropriate depth. A third hole was also drilled and enlarged for placement of an anchor screw (1/8-inch; Small Parts, Miami Lakes, FL). Durelon carboxylate

cement (Norristown, PA) was carefully applied to the exposed cranium, securing both guide cannulae, and encasing the head of the anchor screw. After the dental cement was allowed to harden, the mouse was carefully removed from the stereotaxic apparatus and placed in a standard rat cage, part of which was sitting upon a heating pad (set to low), to recover from the effects of the anesthesia. After about 3-hr, cannulated mice were moved to the colony room. Mice were allowed at least 6 days to recover in the colony room before subsequent microinjections and behavioral testing were performed.

Guide cannulae, stylets, and microinjectors. Guide cannulae, stylets, and tubing to make injectors were special ordered from Small Parts Inc. (Miami Lakes, FL). Guide cannulae were made of 25-gauge stainless-steel tubing, pre-cut to 15.5 mm in length. Stainless-steel wire (0.0095-inch) was used to make stylets; 22 mm segments were fully inserted into single guide cannulae so that one end was flush with the opposite end of the guide cannula. The protruding end of stainless-steel wire was then bent to a 45-degree angle relative to the guide cannula. Stylets were only removed for microinjections, and functioned to keep the guide cannulae free of debris.

Microinjectors were made from two sections of stainless-steel tubing. A 30 mm section of 32-gauge tubing was inserted into a 30 mm section of 25-gauge tubing so that 18 mm of it protruded. Super glue was used to hold the sections in place, forming a microinjector. One end of a 0.5-m length segment of PE-20 tubing was fitted over the 25-gauge end of the microinjector. The opposite end of the PE-20 tubing would ultimately fit over the needle of a glass syringe in preparation for microinjection (see below).

Intra-VTA microinjections. A Cole-Parmer (74900-Series) dual infusion pump was used for all intra-VTA microinjections. Two segments of PE-20 tubing (each attached to a microinjector) were loaded with either 0.9% saline vehicle or baclofen. The other end of the tubing was fitted over the needles of two 0.9% saline-filled 10 µl Hamilton glass syringes. The glass syringes were fitted into the dual syringe pump.

Once the microinjection set-up was complete, the mouse was lightly grasped by the scruff of the neck and the stylets were removed with a pair of forceps. Two microinjectors (attached via the tubing to the glass syringes) were then inserted into/through the guide cannulae. Once fully inserted, the tips of the microinjectors extended 2.5 mm beyond the ends of the guide cannnulae, reaching the VTA.

Saline vehicle or baclofen (200 nl/side) was microinjected at a rate of 382 nl/min. The microinjection took approximately 30 s. After the entire volume had been microinjected, the microinjectors were left in place for an additional 30 seconds to allow for diffusion away from the microinjection tips. Thus, the entire microinjection procedure lasted about 1 min during which time the mouse remained lightly restrained. Upon conclusion of the microinjection procedure, each microinjector was slowly removed to avoid drawing any baclofen back up through the guide cannulae.

Behavioral testing procedure. Behavioral testing occurred on 3 consecutive days. On days 1 and 2, mice were moved to the experiment room, weighed, and allowed to habituate to the testing room environment for at least 60 minutes. Mice were then habituated to the microinjection and behavioral testing procedures. For this, stylets were removed from the guide cannulae, and the microinjectors were inserted. Each mouse was lightly restrained and the microinjectors were left in place for about 60 s, but nothing was

microinjected. The microinjectors were then removed, and each mouse received an IP injection of 0.9% saline and was immediately placed in an automated activity monitor (Accuscan, Columbus, OH) for 30 min. Stylets were re-inserted following each habituation session, and the mouse was returned to its home cage.

On day 3 of the behavioral testing procedure, mice were again moved to the procedure room, weighed, and allowed at least 60 min to habituate to the testing room environment. However, before microinjections commenced, each mouse was randomly assigned to one of 3 baclofen treatment groups (0, 0.01, or 0.02 μg in 200 nl 0.9% saline per side). These doses were chosen based on pilot data that suggested they would have little, moderate, and maximal effects on EtOH stimulated activity. Stylets were again removed, the microinjectors were inserted, and one of the doses of baclofen was bilaterally microinjected into the VTA. To control for any general effects of intra-cranial injections of baclofen, an independent group of FAST-1 and -2 mice received microinjections of 0.02 μg baclofen approximately 2 mm dorsal to the VTA. In most cases these microinjections were into the superior colliculus; the coordinates were, with respect to bregma, 0.5 mm lateral, 0.5 mm posterior, and 2.0 mm ventral. Following microinjection, each mouse received an IP injection of either 2.0 g/kg EtOH (Pharmco Products Inc.; Brookfield, CT; 20% in 0.9% saline), or an equivalent volume of saline, and was immediately placed in the activity monitor for 30 min. FAST mice receiving baclofen 2 mm dorsal of the VTA received only EtOH, and were also immediately placed in the activity monitor. The 30-min time period was chosen based on the time course of baclofen's effects in Experiment 1.

Figure 3. Photomicrographs showing bilateral anterior (panel A) and posterior (panel B) intra-VTA injection sites. Sections were stained with thionin. Arrows indicate injection sites. Abbreviations: AVTA, anterior ventral tegmental area; CA1, field of hippocampus; CA3, field of hippocampus; PAG, periaqueductal gray; PVTA, posterior ventral tegmental area; RMC, red nucleus magnocellular; RPC, red nucleus parvocellular; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata.

Blood EtOH Concentration. Immediately following the final day of behavioral testing, peri-orbital sinus blood (20 µl) was sampled from EtOH-treated mice for determination of BEC as stated above.

Histological verification of injection sites. Following blood sampling for BEC determination, the brain of each mouse was removed and rapidly frozen using cold isopentane (chilled on dry ice) and stored at -80°C. Each brain was then sliced into 50 µm coronal sections using a cryostat (Leica CM1850; Nussloch, Germany), thaw-mounted onto Superfrost Plus, pretreated slides (VWR; West Chester, PA), and stained using thionin (Aldrich Chemical Co.; Milwaukee, WI). Sections were inspected and the injection sites were noted. Photomicrographs illustrating typical posterior and anterior injection sites can be seen in the upper and lower panels of Figure 3, respectively. Mice with microinjection sites located outside the posterior or anterior VTA were excluded from the experimental analysis; 102 out of 418 animals were excluded from the analysis for this reason (overall hit rate of 76%). The locations of injection sites were verified by an independent observer.

Statistical Analysis. Locomotor activity (day 3) and BEC data were analyzed using ANOVA. Simple main effects analysis of significant 2-way interactions and Neuman-Keul's *post-hoc* tests were performed when appropriate. The level of statistical significance was set at $p < 0.05$.

Results

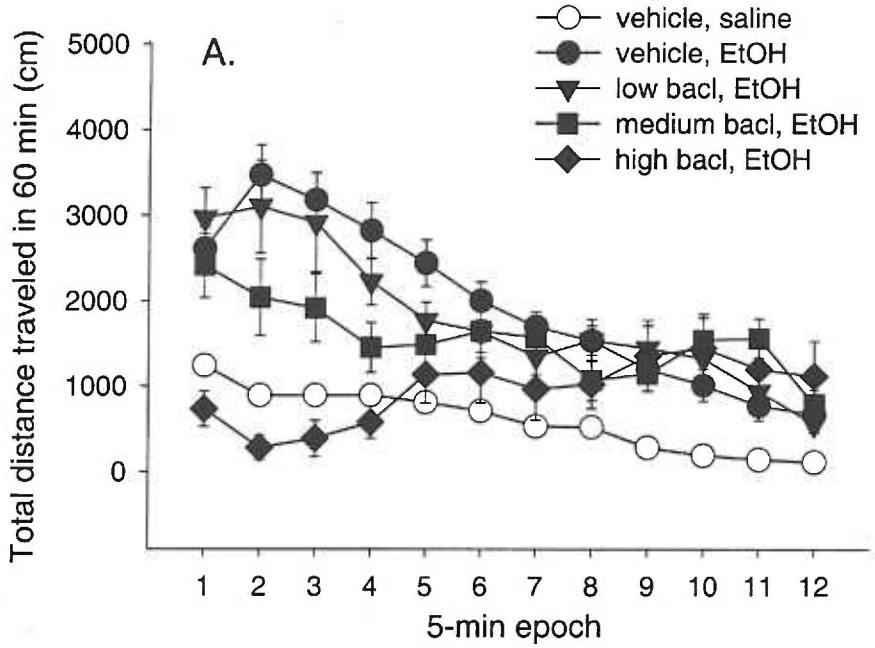
Experiment 1. Intracerebroventricular injections of baclofen.

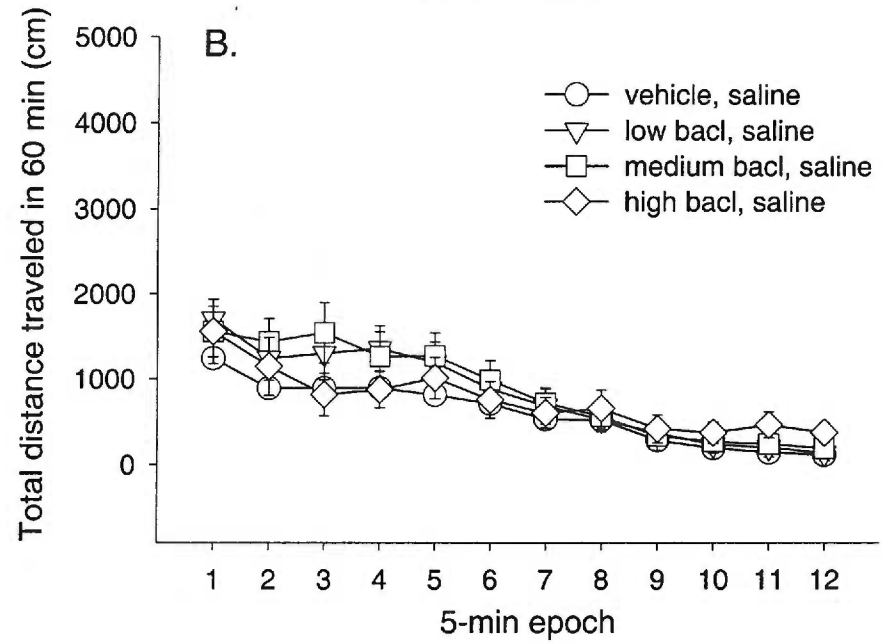
Locomotor activity. Figure 4 (panels A and B) shows time-course data collected begining15 min after ICV injections of baclofen or vehicle in FAST mice. Data are shown collapsed on replicate and sex because these factors did not interact with drug effect factors. We have agreed that this outcome makes analysis collapsed on replicate legitimate and increases the power of the analysis (Crabbe et al., 1990). Inspection of the entire 60-min time-course curve showed that the effects of baclofen waned after the first 30 min. When the time course data for the first 30 min

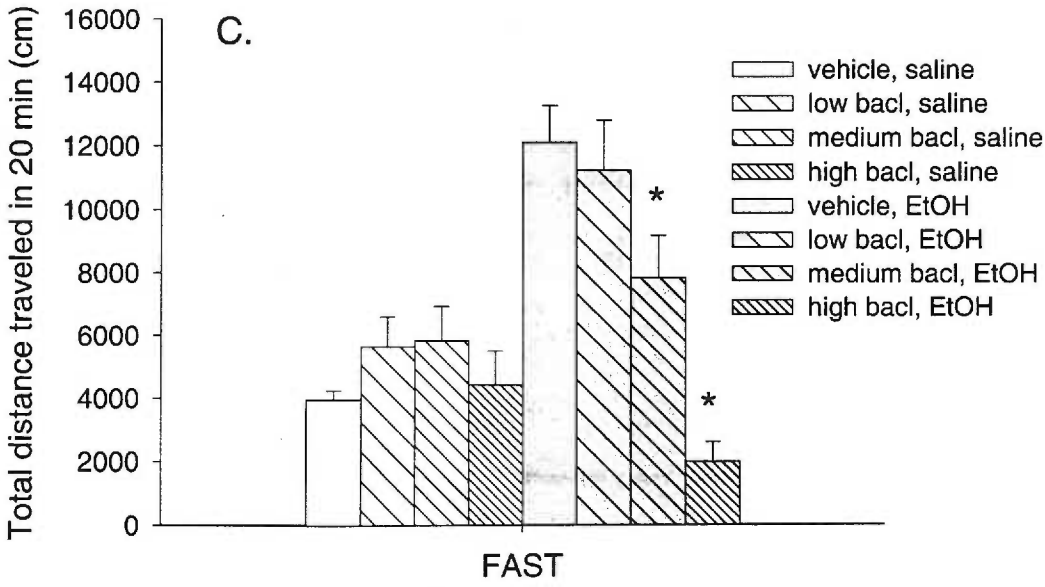
of the locomotor activity test were analyzed by 3-way repeated measures ANOVA, a significant interaction of EtOH, baclofen, and time ($F[15, 780] = 3.2$; $p < 0.001$) was found. Two-way analyses for each 5-min period revealed significant interactions of baclofen and EtOH for the first through fifth intervals of the 60-min test ($F's[3, 156] = 3.8\text{-}9.6$; $p's < 0.02\text{-}0.001$). Thus, to maximize detection of significant drug effects and simplify the analysis, data were summed over the first 20 min of locomotor activity testing for subsequent analyses.

The effects of ICV injection of baclofen on locomotor activity of EtOH- and saline-treated FAST mice during the first 20 min of the test are shown in Figure 4 (panel C). Initially, locomotor activity data following ICV injection were analyzed by a 4-way ANOVA (line x replicate x baclofen dose x EtOH treatment), revealing significant interactions of replicate and EtOH ($F[1, 132] = 4.6$; $p < 0.04$), and baclofen and EtOH ($F[2, 132] = 10.3$; $p < 0.001$). The 4-way interaction was not significant. The replicate x EtOH interaction was likely due to the slightly greater sensitivity to EtOH's stimulant effects in FAST-2 compared to FAST-1 mice. However, because replicate was not involved in other possible interactions, and because sex did not interact with any variable,

Figure 4. ICV baclofen dose-dependently attenuated EtOH's locomotor stimulant effects in FAST mice. Data are shown collapsed on replicate and sex. Doses of baclofen differed slightly for male and female mice due to the greater body weight of male mice; average doses are shown in the figure (actual doses were males: 0.72, 1.44, or 2.88 μg/kg; and females: 0.66, 1.33, or 2.66 μg/kg body weight). For reference, locomotor response to ICV saline is repeated in panels A and B. *Panel A.* ICV baclofen decreased EtOH's locomotor stimulant effects during each of the first five 5-min intervals of the 60-min test period. (p's < 0.02). *Panel B.* ICV baclofen did not alter basal locomotion. *Panel C.* Data were summed over the first 20 min of the 60-min test, the time period when baclofen was maximally effective. Compared to the vehicle-EtOH group, ICV baclofen dose dependently attenuated EtOH's locomotor stimulant effects (* p's < 0.01), but did not alter locomotion in the absence of EtOH.


A.
vehicle, saline
vehicle, EtOH
low bacl, EtOH
medium bacl, EtOH
high bacl, EtOH
Total distance traveled in 60 min (cm)
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12
5-min epoch


B.
vehicle, saline
low bacl, saline
medium bacl, saline
high bacl, saline
Total distance traveled in 60 min (cm)
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12
5-min epoch


C.
vehicle, saline
low bacl, saline
medium bacl, saline
high bacl, saline
vehicle, EtOH
low bacl, EtOH
medium bacl, EtOH
high bacl, EtOH
Total distance traveled in 20 min (cm)
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
FAST

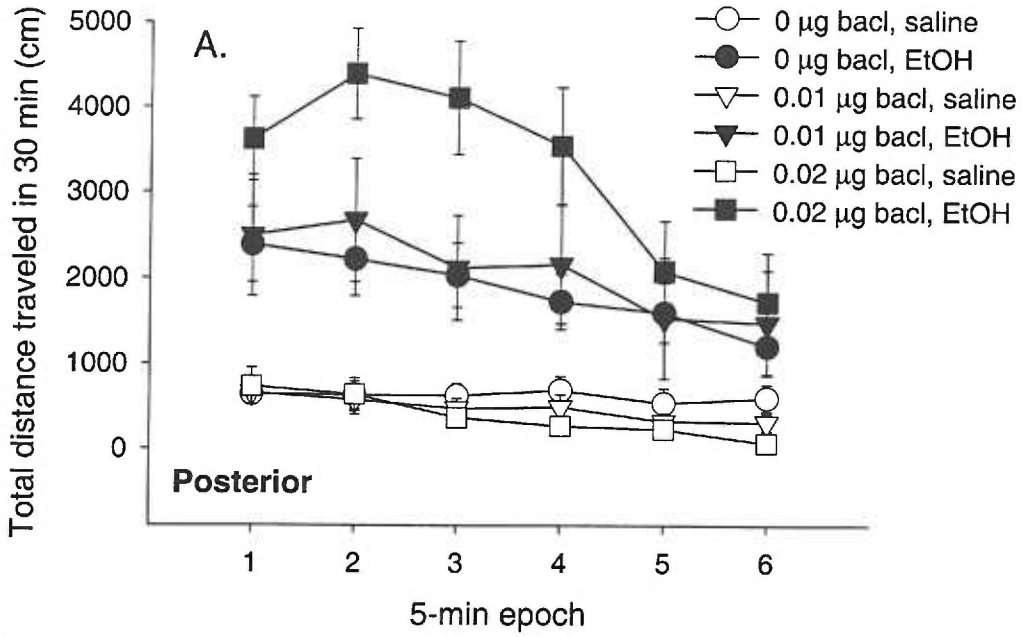
data were collapsed on replicate and sex for further analyses. The baclofen dose x EtOH treatment ANOVA revealed a significant 2-way interaction ($F[2, 156] = 8.8$; $p < 0.001$). EtOH significantly stimulated locomotor activity ($p < 0.001$) and ICV injection of baclofen dose-dependently decreased this EtOH effect. The low dose of baclofen did not significantly alter EtOH stimulation. However, the intermediate and high doses of baclofen significantly attenuated EtOH's stimulant effects (Neuman Keul's *post hoc* test, p's < 0.01). ICV baclofen did not significantly alter locomotor activity in saline-treated FAST mice.

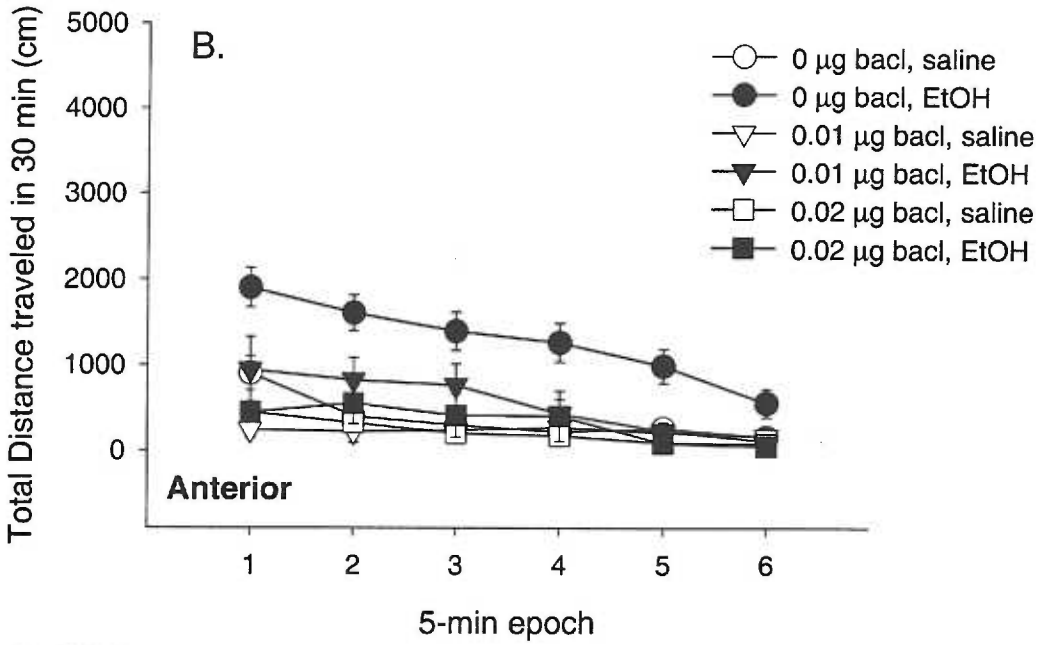
Blood EtOH concentration. Mean BEC (±SEM) was 1.30 ± 0.04 mg/ml, with a range of 1.19 – 1.34 mg/ml. No significant effects of baclofen on BEC were found, nor were there significant differences between males and females, or replicate 1 and 2 mice. These data suggest that ICV baclofen's effects on EtOH-stimulated activity in FAST mice did not result from alterations in EtOH metabolism.

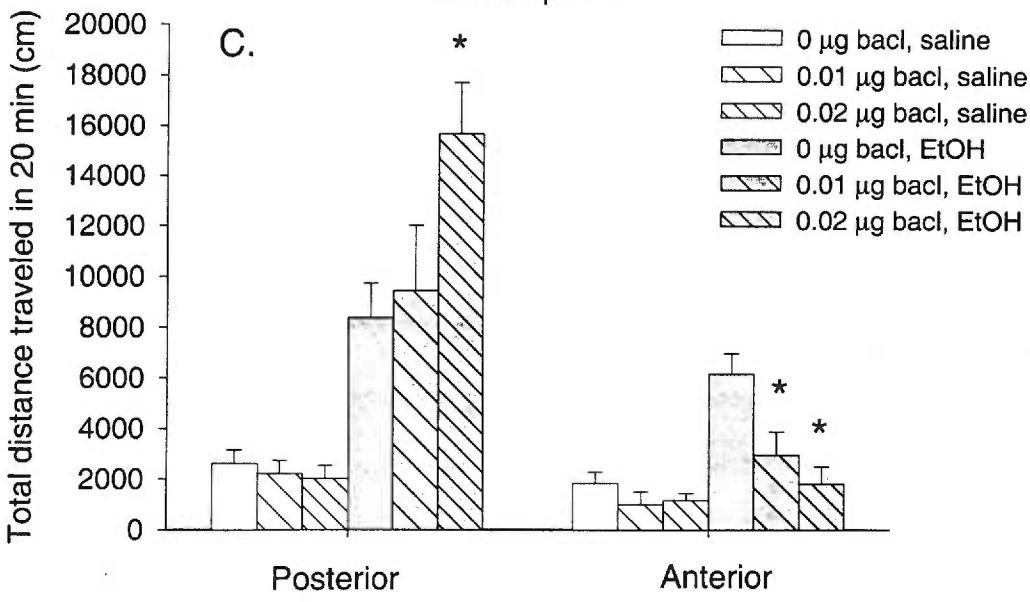
Experiment 2. Intra-VTA microinjections of baclofen.

Locomotor activity. All data were collapsed on replicate for statistical analysis. Based on our knowledge of the variability associated with locomotor behaviors, obtaining group sizes adequate for the analysis of replicate differences (doubling the number of animals) was not deemed feasible. However, inspection of data for each replicate set of lines revealed similar patterns of responses to anterior and posterior intra-VTA baclofen (data not shown). Moreover, when replicate was included in the analysis it did not interact with drug effect factors, suggesting that the patterns of response were similar for the two replicates.

Figure 5. The effects of intra-VTA baclofen in FAST mice depended on region of injection. Data are collapsed on replicate. *Panel A.* The effects of posterior intra-VTA baclofen depended on time ($p < 0.001$); baclofen's effects waned after the first 20 min of the 30-min locomotor activity test session. *Panel B.* The effects of anterior intra-VTA baclofen also depended on time ($p < 0.01$), with baclofen's effects subsiding after the first 25 min of the 30-min test session. *Panel C.* During the time of its maximal effects (first 20 min), posterior intra-VTA baclofen significantly potentiated EtOH's locomotor stimulant effects at the 0.02 μg dose ($p < 0.02$), but not at the 0.01 μg dose. In contrast, anterior intra-VTA microinjection of baclofen blocked EtOH's stimulant effects at both doses (*p's < 0.001).


A.
Total distance traveled in 30 min (cm)
5000
4000
3000
2000
1000
0
Posterior
1
2
3
4
5
6
5-min epoch
0 µg bacl, saline
0 µg bacl, EtOH
0.01 µg bacl, saline
0.01 µg bacl, EtOH
0.02 µg bacl, saline
0.02 µg bacl, EtOH

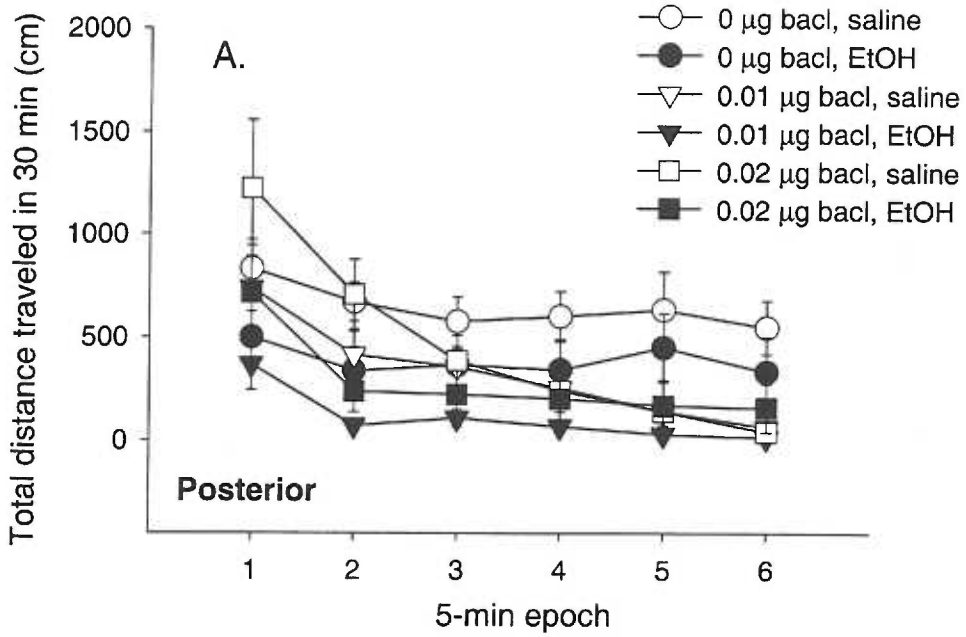

B.
Total Distance traveled in 30 min (cm)
5000
4000
3000
2000
1000
0
Anterior
1
2
3
4
5
6
5-min epoch
0 µg bacl, saline
0 µg bacl, EtOH
0.01 µg bacl, saline
0.01 µg bacl, EtOH
0.02 µg bacl, saline
0.02 µg bacl, EtOH

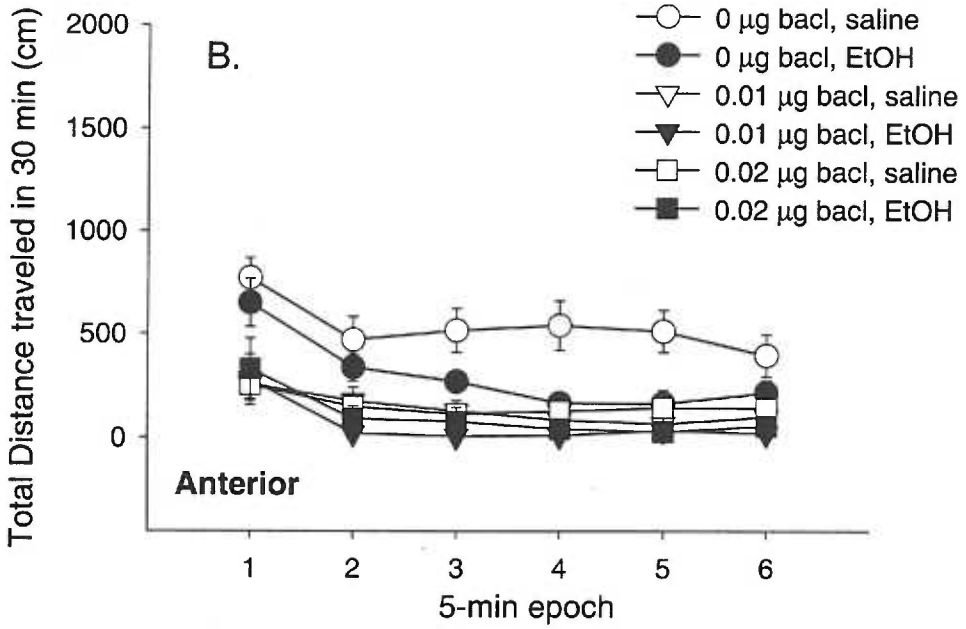

C.
Total distance traveled in 20 min (cm)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
*
Posterior
Anterior
0 µg bacl, saline
0.01 µg bacl, saline
0.02 µg bacl, saline
0 µg bacl, EtOH
0.01 µg bacl, EtOH
0.02 µg bacl, EtOH

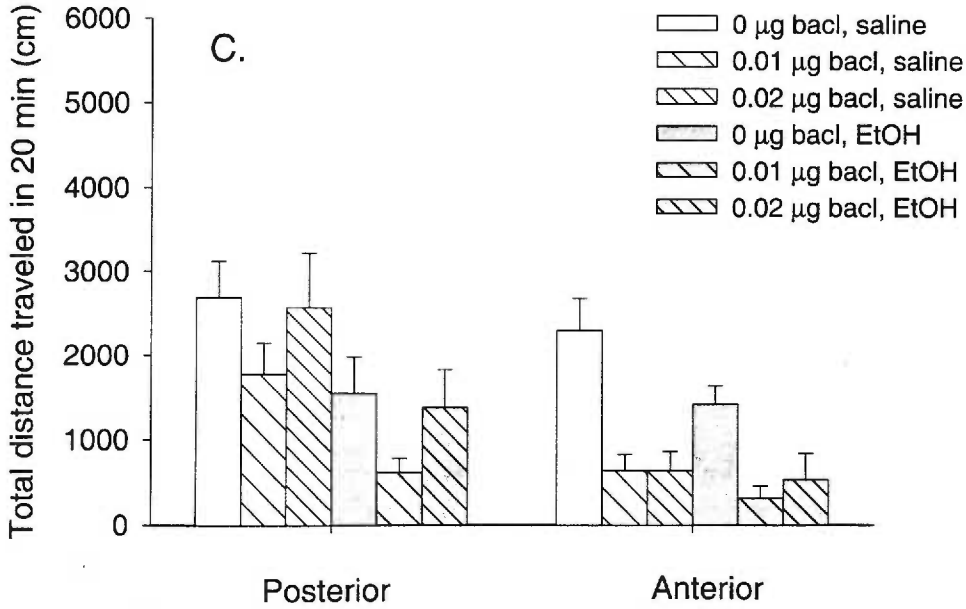
The time-dependent effects of posterior and anterior intra-VTA baclofen in EtOH-treated FAST and SLOW mice are shown in Figures 5 and 6 (panels A and B), respectively. Close inspection of the data revealed that the effects of anterior intra-VTA baclofen waned after the first 25 min of the 30-min test session in both FAST and SLOW mice. Four-way repeated measures ANOVA for anterior intra-VTA data revealed a significant interaction of line, EtOH, and time ($F[5, 725] = 3.6$; $p < 0.01$). Similar time-dependent effects were seen when baclofen was microinjected into the posterior VTA. Four-way repeated measures ANOVA again revealed a significant interaction of line, EtOH, and time ($F[5, 665] = 9.7$; $p < 0.001$). Thus, based on the time course effects of anterior and posterior intra-VTA baclofen, which were similar to those seen with ICV baclofen, only the first four 5-min periods (first 20 min) of the 30-min test session were summed for subsequent statistical analyses.

The effects of anterior microinjection of baclofen into the VTA of EtOH- and saline-treated FAST and SLOW mice for the 20-min testing period can be seen in Figures 5 and 6 (panel C), respectively. Three-way analysis of anterior intra-VTA data revealed a significant interaction of line, baclofen, and EtOH ($F[2, 145] = 4.4$; $p < 0.02$). Subsequent 2-way analyses were performed for each line. For SLOW mice, a significant main effect of baclofen indicated a locomotor depressant effect ($F[2, 67] = 14.7$; $p < 0.001$). SLOW mice also exhibited a reduction in locomotion after EtOH treatment, however, the main effect of EtOH was marginally significant ($F[1, 67] = 3.4$; $p = 0.07$). Thus, anterior intra-VTA microinjection of baclofen decreased locomotion in SLOW mice, but the absence of a significant pre- x post-treatment interaction indicated that this

Figure 6. The effects of intra-VTA baclofen also depended on region of injection in SLOW mice. Data are collapsed on replicate. *Panel A.* Analysis suggested that posterior intra-VTA baclofen depended on time ($p < 0.001$). However, posterior intra-VTA baclofen did not appear to consistently alter locomotor activity in SLOW mice, regardless of EtOH- or saline-treatment. *B.* The effects of anterior intra-VTA baclofen depended on time ($p < 0.01$); baclofen's effects waned after the first 25 min of the 30-min locomotor activity testing session. *Panel C.* During the first 20 min of the test, posterior intra-VTA baclofen did not alter locomotion in SLOW mice, regardless of EtOH- or saline-treatment. However, anterior intra-VTA baclofen had a general locomotor sedative effect not specific to EtOH- or saline-treatment (main effect of baclofen, $p < 0.001$).


A.
Total distance traveled in 30 min (cm)
2000
1500
1000
500
0
Posterior
1
2
3
4
5
6
5-min epoch
0 µg bacl, saline
0 µg bacl, EtOH
0.01 µg bacl, saline
0.01 µg bacl, EtOH
0.02 µg bacl, saline
0.02 µg bacl, EtOH


B.
Total Distance traveled in 30 min (cm)
2000
1500
1000
500
0
Anterior
1
2
3
4
5
6
5-min epoch
0 µg bacl, saline
0 µg bacl, EtOH
0.01 µg bacl, saline
0.01 µg bacl, EtOH
0.02 µg bacl, saline
0.02 µg bacl, EtOH


C.
Total distance traveled in 20 min (cm)
6000
5000
4000
3000
2000
1000
0
Posterior
Anterior
0 µg bacl, saline
0.01 µg bacl, saline
0.02 µg bacl, saline
0 µg bacl, EtOH
0.01 µg bacl, EtOH
0.02 µg bacl, EtOH

effect did not depend on whether the mice had been treated with EtOH or saline (Figure 6, panel C). A similar 2-way analysis for FAST mice did reveal a significant interaction of baclofen and EtOH ($F[2, 78] = 3.9$; $p < 0.03$). Simple main effects analysis showed that EtOH significantly stimulated locomotor activity in FAST mice ($p < 0.001$), consistent with their selected response to EtOH. Neuman-Keul's *post-hoc* tests showed that both doses of anterior intra-VTA baclofen significantly attenuated EtOH's locomotor stimulant effects in FAST mice (p's < .001; Figure 5, panel C). Anterior intra-VTA microinjection of baclofen did not significantly alter locomotor activity in saline-treated FAST mice.

The effects of posterior intra-VTA baclofen in EtOH- and saline-treated FAST and SLOW mice for the 20-min test period are also shown in Figures 5 and 6 (panel C), respectively. Three-way ANOVA of posterior intra-VTA data revealed a significant interaction of line, baclofen, and EtOH ($F[2, 134] = 3.4$; $p < 0.05$). Thus, subsequent 2-way ANOVAs where performed for each line. For SLOW mice, this analysis revealed only a main effect of EtOH ($F[1, 67] = 9.8$; $p < 0.01$); EtOH-treated SLOW mice, regardless of posterior intra-VTA treatment, exhibited lower locomotor activity scores than their saline-treated counterparts (Figure 6, panel C). However, for FAST mice, this analysis revealed a significant interaction of baclofen and EtOH ($F[2, 67] = 3.6$; $p < 0.04$). Simple main effects analysis showed that EtOH significantly stimulated locomotor activity in FAST mice ($p < 0.02$). Moreover, Neuman-Keul's *post-hoc* analysis revealed that for FAST mice, 0.02 μg baclofen, but not 0.01 μg baclofen, significantly enhanced EtOH's locomotor stimulant effects ($p < 0.02$; Figure 5, panel C). Posterior intra-VTA baclofen did not significantly alter locomotion in saline-treated FAST mice.

To test for non-specific effects of intra-cranial injection of baclofen, a separate group of nine EtOH-treated FAST mice received microinjections of 0.02 μg baclofen approximately 2 mm dorsal to the VTA. Control injections of the high dose of baclofen did not significantly alter EtOH's locomotor stimulant effects when compared to EtOH-treated FAST mice that had received an anterior or posterior intra-VTA injection of baclofen, suggesting that the effects of intra-VTA baclofen were specific to the VTA (data not shown).

Blood EtOH concentration. BECs were determined immediately following locomotor activity testing in a subset of mice (n's = 4-12 per line, baclofen dose, and VTA region). Mean BECs (±SEM) following anterior intra-VTA microinjection of 0, 0.01, or 0.02 μg baclofen were 1.81 ± 0.09, 1.58 ± 0.13, and 1.75 ± 0.13 mg/ml for SLOW mice, and 1.65 ± 0.11, 1.95 ± 0.09, and 1.75 ± 0.04 mg/ml for FAST mice, respectively. Two-way ANOVA revealed a significant interaction of line and baclofen ($F[2, 54] = 3.7$; $p < 0.04$). SLOW mice having received intra-VTA injection of 0.01 μg baclofen had significantly lower BEC's than similarly treated FAST mice ($p < 0.02$). However, within each line, intra-VTA baclofen did not significantly alter BEC, suggesting that the behavioral effects of anterior intra-VTA baclofen likely did not result from alterations in EtOH pharmacokinetics.

Posterior intra-VTA microinjections of baclofen did not significantly alter BEC's in either line; mean BECs (±SEM) following posterior intra-VTA microinjection of 0, 0.01, 0.02 μg baclofen were 1.77 ± 0.10, 1.76 ± 0.09, and 1.68 ± 0.11 mg/ml for SLOW mice, and 1.58 ± 0.06, 1.69 ± 0.15, and 1.75 ± 0.04 mg/ml for FAST mice, respectively.

Thus, similar to anterior microinjections, the effects of posterior intra-VTA baclofen are likely not explained by changes in EtOH metabolism.

Discussion

Activation of $GABA_B$ receptors in the brain, in the absence of activation of peripheral $GABA_B$ receptors, attenuated EtOH-induced locomotor stimulation. ICV administration of the $GABA_B$ receptor agonist, baclofen, produced results almost indistinguishable from those seen after systemic baclofen (Shen et al., 1998). Based on the localization of $GABA_B$ receptors to DA cell bodies in the VTA (Grace and Bunney, 1984; Lacey et al., 1988), we hypothesized that activation of $GABA_B$ receptors in this region would attenuate EtOH-stimulated activity. In support of our hypothesis, anterior intra-VTA microinjection of baclofen significantly attenuated or blocked EtOH's locomotor stimulant effects in FAST mice, while having little effect on locomotion in the absence of EtOH. In contrast, posterior intra-VTA injection of baclofen had the opposite effect, significantly enhancing EtOH-stimulated activity in FAST mice, with no effect in non-EtOH treated animals.

That posterior intra-VTA baclofen potentiated EtOH's locomotor stimulant effects in FAST mice was initially surprising. However, this result is consistent with work in rats suggesting regional heterogeneity with regard to VTA function. Anterior, but not posterior, intra-VTA microinjection of both picrotoxin and bicuculline ($GABA_A$ antagonists) stimulated locomotion in rats (Arnt and Scheel-Kruger, 1979). In contrast, microinjection of the $GABA_A$ agonist muscimol stimulated locomotor activity in rats only when it was microinjected into the posterior VTA (Arnt and Scheel-Kruger, 1979;

Wirtshafter and Klitenick, 1989). Self-administration experiments also support regional heterogeneity. Wistar rats readily self-administered picrotoxin to the anterior VTA, but not the posterior VTA, and muscimol to the posterior VTA, but not the anterior VTA (Ikemoto et al., 1997; 1998). Interestingly, one study showed that electrocoagulations of the NAcc resulted in reduced glutamate decarboxylase activity in the anterior, but not posterior VTA, suggesting that the NAcc sends GABA projections to the anterior VTA, but not the posterior VTA (Waalas and Fonnum, 1980). Taken together, these data suggest that the anterior and posterior regions of the VTA differ in anatomical organization of GABA systems.

Recent work has also suggested regional heterogeneity of the VTA with regard to the reinforcing properties of morphine and cocaine in rats (Carlezon Jr. et al., 2000; Rodd-Henricks et al., 2000a). A recent study showed that Wistar rats readily self-administer EtOH into the posterior, but not anterior VTA (Rodd-Henricks et al, 2000b), suggesting that the posterior region of the VTA mediates EtOH's rewarding properties. The parallelism of our locomotor stimulant results with those for EtOH self-administration are striking, and appear to support the notion of common neural circuitry for both EtOH's stimulant effects and EtOH reward. The current results suggest that FAST mice would readily self-administer EtOH to the posterior VTA, but not the anterior VTA.

SLOW mice were included in the present experiments when we began detecting the potentiating effect of baclofen in the posterior VTA. We hypothesized that insensitive SLOW mice would exhibit a stimulant response to EtOH when baclofen was specifically infused into this region. However, posterior intra-VTA baclofen had no

effect in SLOW mice, regardless of EtOH- or saline-treatment. Anterior intra-VTA baclofen did have a general locomotor sedative effect in both EtOH- and saline-treated SLOW mice. Considered along with previously published results showing that SLOW-1 mice were more sensitive than FAST-1 mice to the locomotor sedative effects of systemic baclofen (Shen et al, 1998), the present data suggest that the density of $GABA_B$ receptors in the anterior region of the VTA of SLOW mice may have increased as a result of selection, relative to FAST mice. However, this picture may be too simplistic, as several groups have revealed the existence of the $GABA_{B1a}$ and $GABA_{B1b}$ (Kaupmann et al, 1997), and more recently, the $GABA_{B2}$ (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999) receptor subunits. Evidence suggests that functional $GABA_B$ receptors consist of a heterodimer, comprised of one of the $GABA_{B1}$ receptor splice variants, and the $GABA_{B2}$ receptor subunit. Thus, FAST and SLOW mice may instead differ in $GABA_B$ receptor subunit composition of the anterior VTA. Autoradiography is currently under way in our laboratory to address the possibility that FAST and SLOW mice differ in anterior $GABA_B$ receptor density. Techniques such as *in situ* hybridization will be necessary to elucidate whether the line differences in baclofen sensitivity can be attributed to differing $GABA_B$ receptor subunit composition.

The site of baclofen microinjection was histologically verified. However, we cannot be certain of the extent of baclofen's diffusion away from the site of injection. Our control experiment in which 0.02 μg baclofen was microinjected 2 mm dorsal to the VTA argues against significant diffusion of baclofen away from the injection site. Still, it remains possible that baclofen altered EtOH's locomotor stimulant effects by diffusing to a neural structure located close to the VTA. Rat studies suggest the existence of $GABA_B$

receptors in the substanta nigra pars compacta (Bowery et al., 1987; Chu et al., 1990; Kaupmann et al., 1997), a structure located just ventrolateral to the VTA. This structure is thought to have a role in mediating general locomotion. $GABA_B$ receptor autoradiography will not resolve this issue. However, it will reveal $GABA_B$ receptor rich areas near the VTA that represent potential targets for future investigations to verify (or refute) that the current effects are VTA-mediated.

There are several possible explanations for baclofen's region-specific effects in the VTA. One possibility is that in the anterior VTA, $GABA_B$ receptors are localized on the DA cell bodies themselves, so that baclofen exerts a direct inhibition of EtOH's stimulation of DA release. In contrast, posterior VTA $GABA_B$ receptors may be localized on GABA interneurons that synapse on the DA cell bodies, resulting in an inhibition of the GABA interneurons in the presence of baclofen, and subsequent disinhibition of the DA cell bodies. $GABA_B$ receptor autoradiography is not likely to provide insight into cell body versus interneuron localization. However, $GABA_B$ receptor binding should be seen in both the anterior and posterior regions of the VTA, making this hypothesis tenable. Future studies might utilize immuno-labeling techniques to visualize $GABA_B$ receptors, as well as DA and GABA containing neurons, to elucidate differences in $GABA_B$ receptor localization between anterior and posterior regions of the VTA.

The VTA also sends GABA projections to the NAcc (Van Bockstaele and Pickel, 1995). Interestingly, a recent study showed that these GABA projections may represent a separate neural circuit that, like the DA projections to the NAcc, appear to mediate drug reward (Laviolette et al., 2001). Using a conditioned place preference paradigm, these

authors demonstrated that intra-VTA microinjection of both an agonist and antagonist of the $GABA_A$ receptor in rats resulted in a preference for the drug-paired location. Furthermore, these effects could be dissociated by co-microinjection of a non-selective DA receptor antagonist, suggesting the existence of both DA dependent and independent reward pathways. This study did not detect a difference in anterior-posterior sensitivity within the VTA. However, collectively, these studies suggest another explanation for the region-specific effects of intra-VTA baclofen; these effects could be the result of pharmacological manipulation of two independent neural circuits. For example, in the anterior region, $GABA_B$ receptors may reside on the DA cell bodies where they directly hyperpolarize them. However, in the posterior VTA, $GABA_B$ receptors may be localized on GABA neurons projecting to the NAcc. Given this scenario, $GABA_B$ receptor stimulation would directly hyperpolarize the GABA projection neurons, disinhibiting post-synaptic neurons in the NAcc, resulting in enhanced firing of those neurons, and presumably, a potentiated locomotor stimulant response to EtOH.

Baclofen has been studied in the clinical setting as a potential treatment for the abuse of psychostimulants. Data suggest that baclofen may be of some therapeutic value since it appears to attenuate the subjective feelings of craving in cocaine, EtOH, and tobacco abusers (Ling et al., 1998; Addolorato et al., 2000; Cousins et al, 2001). Given evidence that EtOH craving or reward appear to be influenced by the VTA, it may at first seem alarming that baclofen would have region-specific effects on EtOH-stimulated activity. However, the effects of systemic and ICV baclofen still support its overall attenuating action. Moreover, at the regional level, anterior effects of intra-VTA baclofen may over-shadow the posterior effects, resulting in a net attenuation of EtOH's

locomotor stimulant effects. Thus, our data suggest that $GABA_B$ receptors in the anterior VTA of FAST mice may ultimately mediate the locomotor output of the system, even when posterior $GABA_B$ receptors are also occupied, such as with systemic and ICV administration of the drug just prior to EtOH injection. That SLOW mice are generally more sensitive to anterior intra-VTA baclofen also supports a dominant role for $GABA_B$ receptors in this region of the VTA; SLOW mice are resistant to EtOH's locomotor stimulant effects, suggesting that $GABA_B$ receptors in this region of the VTA render the DA cell bodies incapable of increased firing in response to EtOH.

To conclude, we have shown that $GABA_B$ receptors in the anterior VTA can mediate baclofen's attenuating effects on EtOH-stimulated activity. Moreover, regional heterogeneity of the VTA, similar to that seen for EtOH self-administration in rats, also generalizes to EtOH's locomotor stimulant effects in mice. Taken together, these data suggest that the anterior and posterior regions of the VTA may be structurally different, leading to differing behavioral outcomes when either region is manipulated relative to the other. Whereas, our data suggest an important $GABA_B$ component, future studies will be necessary to elucidate the neurocircuitry, neurochemistry, and neurophysiology underlying these genetic differences. Moreover, the ability to regionally differentiate effects within the extremely small VTA of the mouse holds promise for future explorations of this genetically rich species.

CHAPTER 2

$GABA_B$ receptor densities in the ventral tegmental area and substantia nigra pars compacta of FAST and SLOW mice.

Introduction

The development of medical treatment strategies for alcoholism depends upon the identification of biological risk factors. Clincial studies suggest that one of these risk factors may be sensitivity to alcohol's locomotor stimulant effects (Newlin and Thomson, 1999; Poikolainen, 2000; Holdstock et al., 2000). Pre-clinical studies provide additional support. Studies in mice have consistently shown that sensitivity to this alcohol (ethanol; EtOH) effect is genetically influenced (Crabbe et al., 1987; Dudek et al., 1990; Crabbe et al., 1994; Shen et al., 1995). Furthermore, existing evidence suggests that EtOH's locomotor stimulant and rewarding effects may be mediated by similar biological mechanisms (Kalivas et al., 1990; Amalric and Koob, 1993; Hooks and Kalivas, 1995). Thus, a better understanding of the biological mechanisms that mediate EtOH's stimulant effects may ultimately help us understand the biological mechanisms leading to alcohol abuse and addiction.

It is believed that the locomotor stimulant effects of EtOH are partly mediated by the mesoaccumbens-pallidal circuit (Amalric and Koob, 1993; Hooks and Kalivas, 1995; Kalivas and Nakamura, 1999). The principal components of this circuit are dopamine (DA) neurons that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc). The DA neurons synapse on γ-aminobutyric acid (GABA) neurons

in the Nacc, which project to the ventral pallidum/substantia innominata, where they modulate output to the extrapyramidal system. Similar to that of other abused drugs, EtOH administration increases DA release from DA nerve terminals in the Nacc (Imperato et al., 1987; Kalivas and Stewart, 1991; Smith and Weiss, 1999).

$GABA_B$ receptors are G-protein (G*i*) coupled receptors that inhibit adenylate cyclase (Wojcik and Neff, 1984; Hill, 1985; Karbon and Enna, 1985; Andrade et al., 1986). These receptors are known to reside in the VTA were they modulate activity of the mesolimbic DA circuit (Grace and Bunney, 1984; Lacey et al., 1988; Kalivas et al., 1990). Furthermore, pharmacological studies in mice have shown that $GABA_B$ receptor agonists and antagonists modulate ethanol-stimulated locomotion (Allan and Harris, 1989; Humeniuk et al., 1993). Through genetic selection, we have generated replicate mouse lines that differ in sensitivity to EtOH-stimulated activity (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995). FAST-1 and –2 mice exhibit extreme sensitivity to EtOH's locomotor stimulant effects, whereas SLOW-1 and –2 mice are resistant to these effects. These lines provide a powerful means by which to investigate $GABA_B$ receptor modulation of EtOH-stimulated activity.

We have investigated the effects of the $GABA_B$ receptor agonist, baclofen, in the FAST and SLOW selected lines. When administered systemically, baclofen dose-dependently attenuated EtOH's locomotor stimulant effects in FAST mice (Shen et al., 1998). Moreover, when baclofen was administered in the absence of EtOH, SLOW-1 mice exhibited a greater general locomotor sedative effect than FAST-1 mice; FAST-2 and SLOW-2 mice did not significantly differ. Similar results were seen when baclofen was intracerebroventricullarly (ICV) administered to FAST and SLOW mice (Boehm II

et al., *submitted*). These results offer evidence of an important role for $GABA_B$ receptors in mediating EtOH's stimulant effects in FAST mice, and also suggest that genetic selection for differential sensitivity to EtOH's locomotor stimulant effects might have altered $GABA_B$ receptor density and/or function, or perhaps neurocircuitry influenced by $GABA_B$ receptor activation (see Crabbe et al., 1990 for a review on interpreting selected line data).

Our ICV baclofen data suggest that activation of central $GABA_B$ receptors could inhibit EtOH-stimulated locomotion. To test the hypothesis that baclofen's effects on EtOH-stimulated activity are mediated specifically by $GABA_B$ receptors in the VTA we performed a microinjection study. Baclofen was injected directly into the VTA of FAST and SLOW mice (Boehm II et al., *submitted*). In FAST mice, anterior intra-VTA baclofen blocked the locomotor stimulant effects of EtOH. In contrast, posterior intra-VTA baclofen enhanced EtOH-stimulated activity in FAST mice. Intra-VTA baclofen had no effect in FAST mice when it was microinjected in the absence of EtOH. However, SLOW mice displayed a marked general locomotor sedative effect when baclofen was microinjected into the anterior region of the VTA. Importantly, this effect was not apparent when baclofen was microinjected into the posterior VTA. Considered along with the replicate 1 difference seen following systemic administration of baclofen, these data offer strong evidence that genetic selection for differential sensitivity to EtOH's locomotor stimulant effects resulted in line differences in $GABA_B$ receptor density, function, or influence in the anterior region of the VTA.

The current study investigated $GABA_B$ receptor density. $GABA_B$ receptor autoradioagraphy was used to determine the density of $GABA_B$ receptors in the anterior

and posterior regions of the VTA in FAST and SLOW mice. We predicated that compared to FAST mice, SLOW mice would exhibit a higher density of $GABA_B$ receptors in the anterior, but not posterior VTA. We also examined $GABA_B$ receptor density in the substantia nigra pars compacta (SNC) of FAST and SLOW mice. This structure is located just ventrolateral to the VTA, and is known to have a role in mediating locomotion. Thus, the SNC may have been an alternative site of baclofen's effects in the previously described intra-VTA microinjection experiment.

Methods

Subjects.

The FAST and SLOW mouse lines were selectively bred in replicate for differential sensitivity to EtOH-stimulated locomotor activity. FAST-1 and –2 mice exhibit extreme sensitivity to EtOH's locomotor stimulant effects, whereas SLOW-1 and –2 mice mice are resistant to this effect (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995). Mice were selectively bred based on locomotor response to EtOH administration, corrected for locomotor response to saline administration indexed 24 hours later. Locomotor activity was measured in Lehigh Valley circular open-field activity monitors. The highest scoring male and female from each of 9 families per replicate line was mated in a rotational within-family breeding scheme to produce FAST-1 and -2 offspring for the next generation. Likewise, mice exhibiting extremely low scores were mated to produce SLOW-1 and -2 offspring. Brother-sister mating was prohibited. Selective breeding was relaxed after 37 generations, and the lines are now maintained at the Portland VA Medical Center. Mice for the current studies were from

second or later litters of the primary FAST and SLOW breeders. They were weaned at 21±2 days of age and housed 3-4 per cage with mice of the same sex and genotype. Clear polycarbonate cages (28 by 18 by 13 cm) with corn-cob bedding were used, and mice had free access to standard rodent chow and water. Mice were maintained on a 12-hr light-dark cycle (lights on at 0600 hours) and an ambient room temperature of 22 ±2°C. All procedures were approved by the Portland Veterans Affairs Medical Center Institutional Animal Care and Use Committee, and conformed with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals.*

Drugs.

2-Hydroxysaclofen was obtained from Sigma Aldrich (St. Louis, MO). [^{3}H]CGP 54626 (specific activity 40 Ci/mmol) was obtained from Tocris Cookson (Bristol, U.K.). [^{3}H]CGP 54626 was chosen for these experiments based on its high affinity for the $GABA_B$ receptor (Green et al., 2000). Moreover, the pattern of $GABA_B$ receptor autoradiography in rat brain is similar to that seen using ^{3}H-baclofen (Bischoff et al., 1999).

In vitro [^{3}H]CGP 54626A binding assay.

Membrane Preparation. Prior to autoradiographic binding experiments, in vitro [^{3}H]CGP 54626A binding in male FAST-2 cortex was performed to establish a K_D for the compound. Male FAST-2 mice were chosen because they were abundant in our breeding colony at the time this experiment was performed. Mice were euthanized, and their cortices were rapidly dissected and kept on ice. Cortical tissue was homogenized in 50 volumes of ice-cold 50 mM Tris-Citrate buffer (pH 7.1), and spun at 48,000 x g for 10 min (4°C). This process was repeated 5 additional times, except that 50 volumes of ice-

cold 5 mM Tris-Citrate buffer (pH 7.1) replaced the original buffer after the second, fourth, and sixth spins. The lower concentration buffer was used to more efficiently remove endogenous GABA by osmotically shocking the membranes. Following the sixth spin, the membranes were resuspended and frozen at –80°C overnight. The next day, membranes were thawed, and the previous day's membrane preparation process was continued; membranes were homogenized in ice-cold 50 mM Tris-Citrate buffer (pH 7.1) following the first spin, and in 5 mM Tris-Citrate buffer (pH 7.1) following the second spin. Following the third spin, pellets were resuspended in 50 volumes ice-cold Krebs-Henseleit assay buffer (120 mM NaCl, 6 mM glucose, 20 mM Tris, 4.7 mM KCl, 1.8 mM $CaCl_2$ $2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, pH 7.4), separated into 2 ml aliquots, and frozen at –80°C until use.

Binding assay. The [^{3}H]CGP 54626 binding assay was first described by Bischoff et al. (1999). Membranes were thawed in a 25°C water bath. For radioligand binding, 50-100 µg membrane protein was incubated in 200 µl Krebs-Henseleit assay buffer (pH 7.4) and 50 µl 0.375, 0.75, 1.5, 3.0, 6.0 or 12.0 nM [^{3}H]CGP 54626, at 22±1°C for 20 min. Non-specific binding was determined in the presence of 1 mM 2-hydroxysaclofen. Incubation was terminated by rapid filtration through a 96-well plate Harvester (Tomtec; Hamden, CT). The amount of filter-bound radioactivity was assessed by a Wallac 1205 Betaplate liquid scintillation counter (Turku, Finland). The assay was repeated 3 times using different FAST-2 mouse cortical preparations, and the total amount of protein was determined by the Pierce BCA protein assay (Rockford, Il). Binding curves and K_D and Bmax values were generated using GraphPad Prizm 3 software (San Diego, CA).

$GABA_B$ receptor autoradiography.

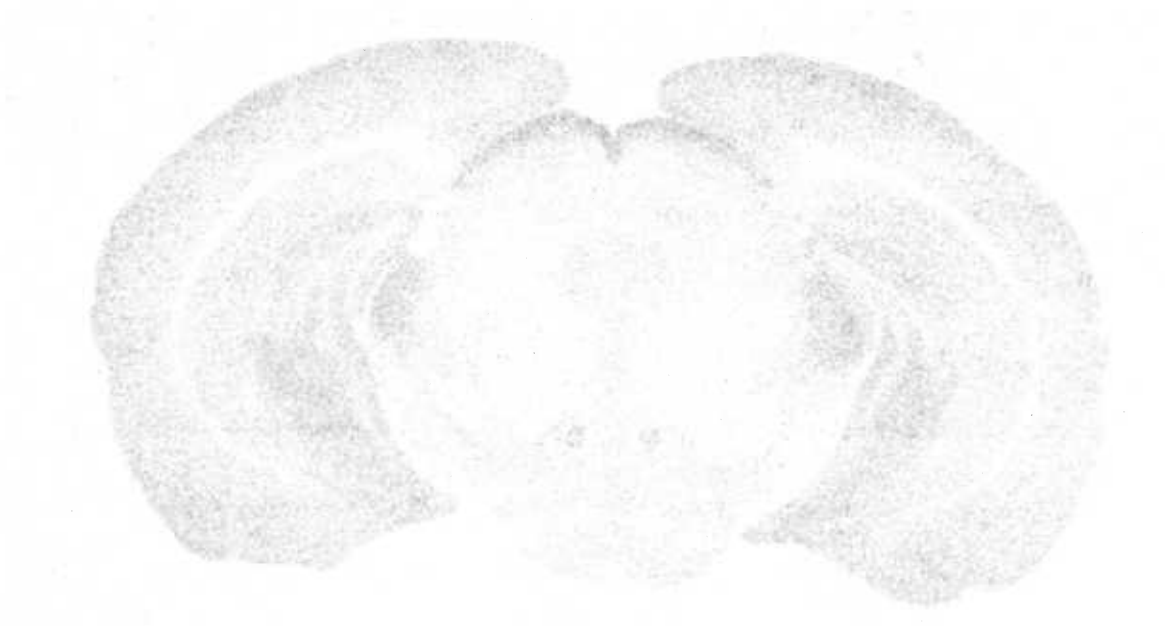
Brain slice preparation. Male FAST-1 and -2 and SLOW-1 and -2 mice (3 per line and replicate, except for FAST-2 mice of which there were 4) were euthanized and the brain of each mouse was removed and rapidly frozen using cold isopentane (chilled on dry ice) and stored at -80°C until sectioning. Each brain was sliced into 16 μm coronal sections using a cryostat (Leica CM1850; Nussloch, Germany), thaw-mounted onto cold Superfrost Plus pretreated slides (VWR; West Chester, PA), and stored at -80°C until assay. Sections were cut at the level of the VTA and SNC. Three adjacent brain slices were mounted on 3 consecutive slides. The first slide was subsequently subjected to an analysis of specific binding, the second to a non-specific binding analysis, and the third to thionin staining for histological verification of location in the mouse brain.

Autoradiography assay. The autoradiographic assay was adapted from that of Bischoff et al. (1999). Slides were thawed and dried at room temperature for 1-2 hr, and then preincubated in Krebs-Henseleit assay buffer (pH 7.4; 22±1°C) for 15 min. Total binding was achieved by incubating the appropriate slides in 2 nM [^{3}H]CGP 54626 in Krebs-Henseleit assay buffer (pH 7.4; 22±1°C) for 2 hr. Non-specific binding was assessed by incubating the appropriate slides in the above along with 200 μM 2-hydroxysaclofen. To terminate binding, slides were washed twice for 8 sec in Krebs-Henseleit assay buffer (pH 7.4; 4°C), dipped in ice-cold water, and dried at -20°C overnight. Slides were exposed to tritium-sensitive Hyperfilm (Amersham; Amersham, U.K.) for 5 days.

Figure 7. Corresponding mouse brain slices showing non-specific (upper panel), and total (lower panel) [^{3}H] CGP 54626 binding. Non-specific binding was achieved in the presence of 2-hydroxysaclofen. Non-specific binding was subtracted from total binding to get an estimate of specific [^{3}H] CGP 54626 binding.

Non-Specific Binding

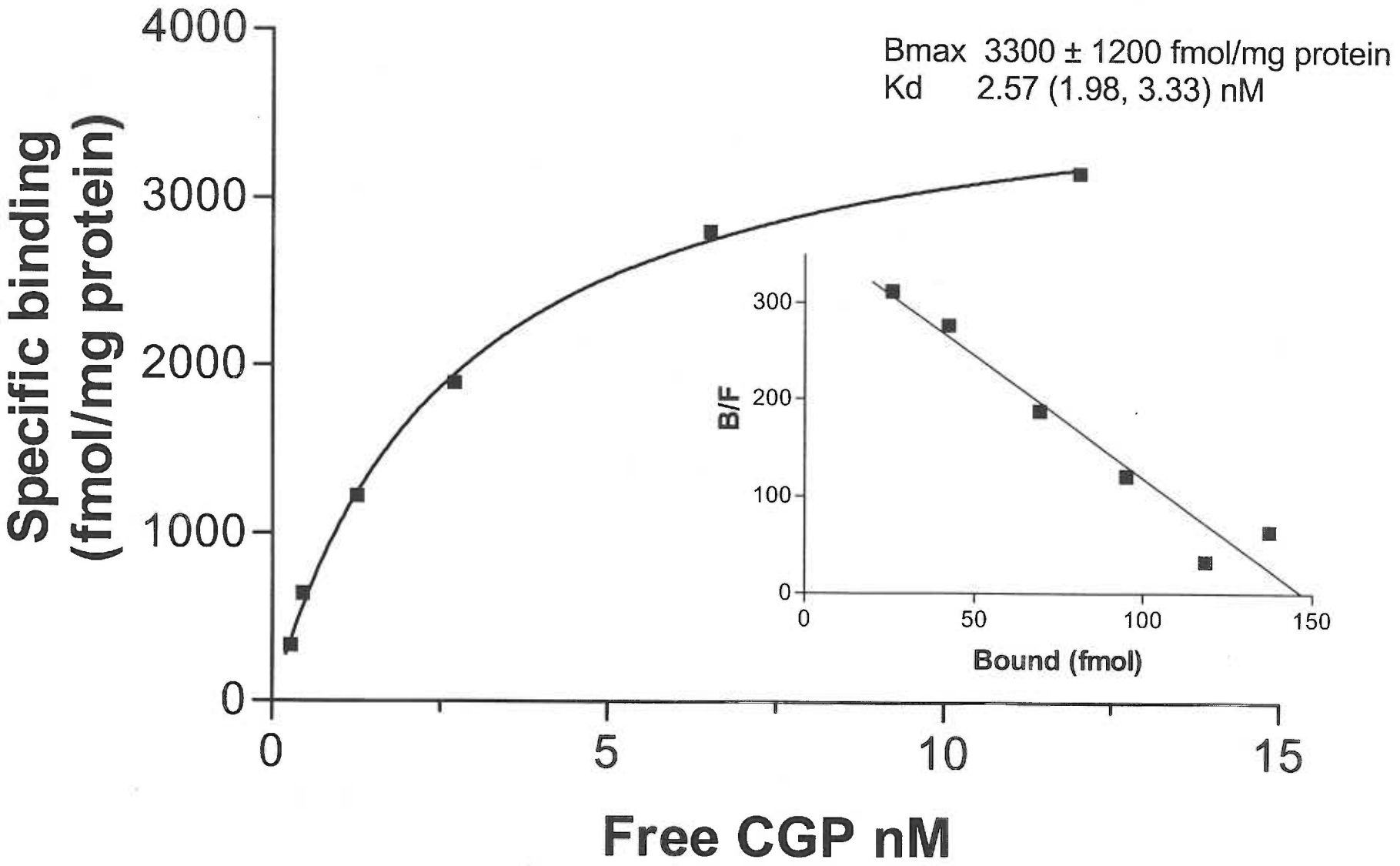
Total Binding



Quantitative analysis was performed using the Optiquant Verson 3.0 image-processing program (Meriden, CT), and involved assessing optical densities of film gray values at approximately 64 μm intervals anterior to posterior through the VTA and SNC. Optical densities of film gray values assessed by the Optiquant program were converted to bound radioactivity using tritiated calibration markers (RPA 506; Amersham), and were within the linear range of the standard curve. Data are expressed as fmols/mg tissue. Four separate assessments of binding density were made for each brain slice and neural structure. An assessment of background binding density was also made for each brain slice, and subtracted from the four measured densities of each neural structure. The corrected binding densities were then averaged to give a single estimate of [^{3}H] CGP 54626 binding for each neural structure and brain slice. Finally, the binding densities from brain slices subjected to non-specific binding were subtracted from those of the corresponding brain slices subjected to total binding, giving an estimate of specific [^{3}H] CGP 54626 binding. Figure 7 shows examples of two corresponding mouse brain slices, one subjected to non-specific binding, and the other subjected to total binding.

In the intra-VTA microinjection experiment shown in Chapter 1, data were collapsed on replicate largely because it was not possible to include enough animals of each replicate for a statistically reliable evaluation. Due to similar constraints, and because we wanted to assess VTA and SNC $GABA_B$ receptor densities based on the results reported in Chapter 1, the current data set was also collapsed on this factor for statistical analysis. Data were analyzed using analysis of variance (ANOVA). The level of significance was set at $p < 0.05$.

Figure 8. [^{3}H]CGP 54626 saturation binding to FAST-2 cortical membranes. The binding assay was repeated 3 times. A representative binding curve is shown. The geometric mean K_D and the arithmetic mean Bmax values of the compound were 2.76±0.65 nM and 3,300±1,200 fmols/mg protein, respectively. The insert shows the Scatchard transformation and analysis for the saturation curve. The linearity of the Scatchard plot is indicative of a single $GABA_B$ receptor binding site for [^{3}H]CGP 54626.


Bmax 3300 ± 1200 fmol/mg protein
Kd 2.57 (1.98, 3.33) nM
Specific binding (fmol/mg protein)
4000
3000
2000
1000
0
0
5
10
15
Free CGP nM
B/F
300
200
100
0
0
50
100
150
Bound (fmol)

Results

In vitro [^{3}H]CGP 54626A binding assay.

An assessment of [^{3}H]CGP 54626 binding in the cortical membranes of FAST-2 mice was made to establish the binding parameters of the compound in our hands. The binding assay was repeated 3 times, and the geometric mean of K_D and arithmetic mean of Bmax values were calculated. The average K_D value for [^{3}H]CGP 54626 was estimated at 2.76±0.65 nM, and average Bmax at 3,300±1,200 fmols/mg protein. A representative binding curve can be seen in Figure 8. These data were used to establish the parameters of the [^{3}H]CGP 54626 autoradiography assay.

$GABA_B$ receptor autoradiography.

Ventral Tegmental Area (VTA). The pattern of $GABA_B$ receptor binding through the VTA of FAST and SLOW mice can be seen in Table 2, and shown graphically in Figure 9. $GABA_B$ receptor densities varied from anterior to posterior through the VTA for both lines. Based on the behavioral results of anterior and posterior intra-VTA microinjection of baclofen (Chapter 1), we predicted that SLOW mice might possess a greater $GABA_B$ receptor density in the anterior region of the VTA, compared to FAST mice. Furthermore, we predicted that $GABA_B$ receptor density in the posterior region of the VTA would not differ between the lines. Initial one-way analysis of data summed and averaged across the entire VTA (-2.92 to -3.88 mm, with respect to bregma) of each mouse did not reveal a significant difference between the FAST and SLOW mice (223±19 and 183±16 fmols/mg tissue for FAST and SLOW mice, respectively; $F[1, 11] = 2.34$, $p = 0.15$). Likewise, when data for each mouse were summed, and then averaged

Table 2. Density of $GABA_B$ Binding Sites Through the Ventral Tegmental Area and Substantia Nigra Pars Compacta of FAST and SLOW Mice[1].

	VTA		SNC	
Neural Region[2]	FAST	SLOW	FAST	SLOW
Anterior				
-2.92	129±13	138±30	165±28	180±23
-3.08	252±32	143±32	264±22	192±34
-3.16	242±34	171±22	262±22	225±25
-3.28	256±32	258±39	260±30	280±38
-3.4	243±29	213±56	239±29	217±55
Posterior				
-3.52	254±33	195±33	263±24	224±36
-3.64	233±27	165±37	228±35	200±42
-3.8	191±14	201±36	194±23	211±33
-3.88	226±27	165±18	212±23	147±16

[1]Values represent fmols [^{3}H]CGP 54626 bound per mg tissue.

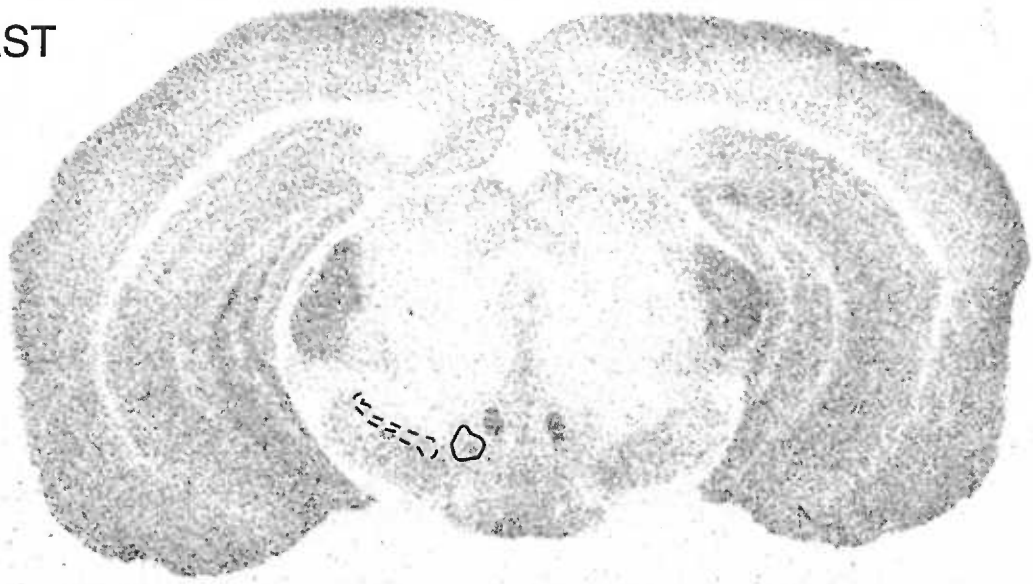
[2]Each neural region corresponds to a panel found in the mouse brain atlas of Franklin and Paxinos (1997), and is given in mm with respect to bregma.

Figure 9A-I. Representative photomicrographs showing the pattern of $GABA_B$ receptor densities through the ventral tegmental area and substantia nigra compacta of FAST (upper panels) and SLOW (lower panels) selectively bred mice using [^{3}H]CGP 54626. Coronal sections correspond to the coordinates of the published panels in the mouse brain atlas of Franklin and Paxinos (1997). The measurements in the upper left corner of each panel refer to mm with respect to bregma. Solid lines indicate the VTA, whereas dashed lines indicate the SNC.

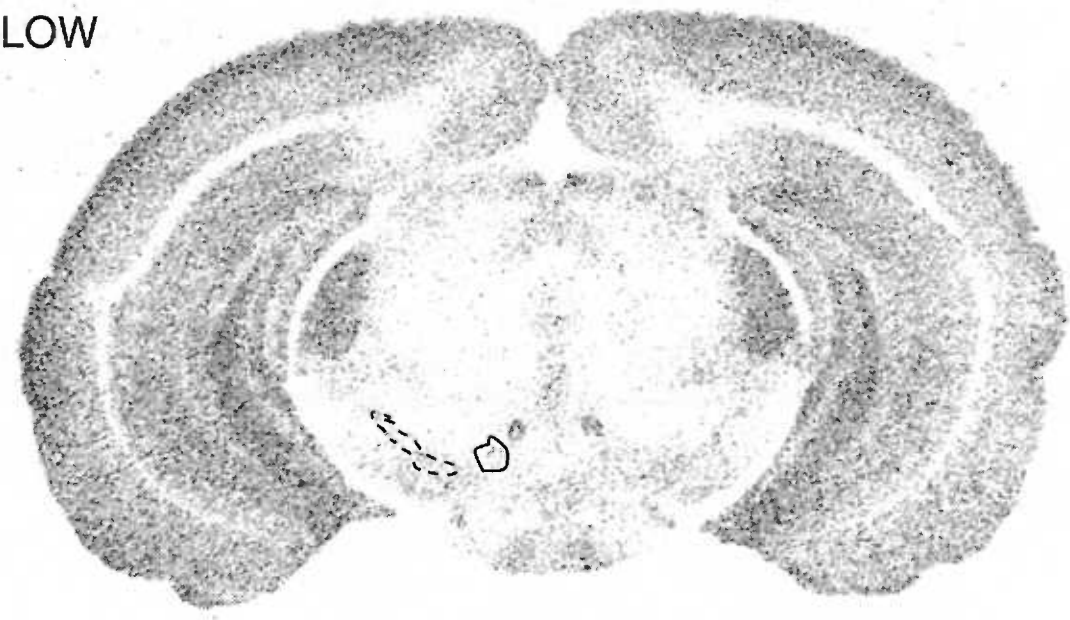
A.

-2.92 mm

FAST



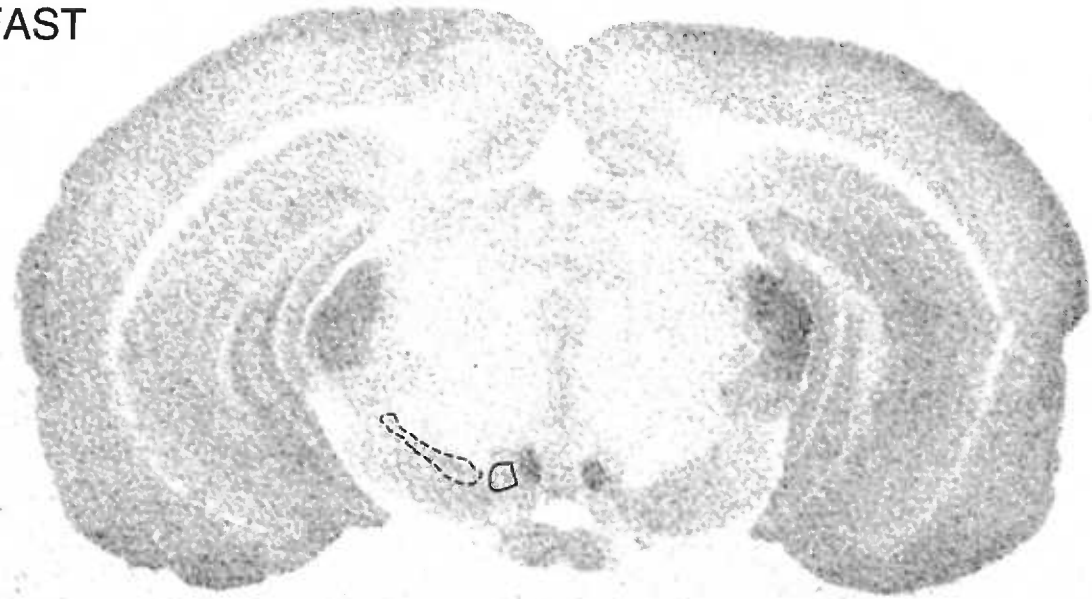
SLOW



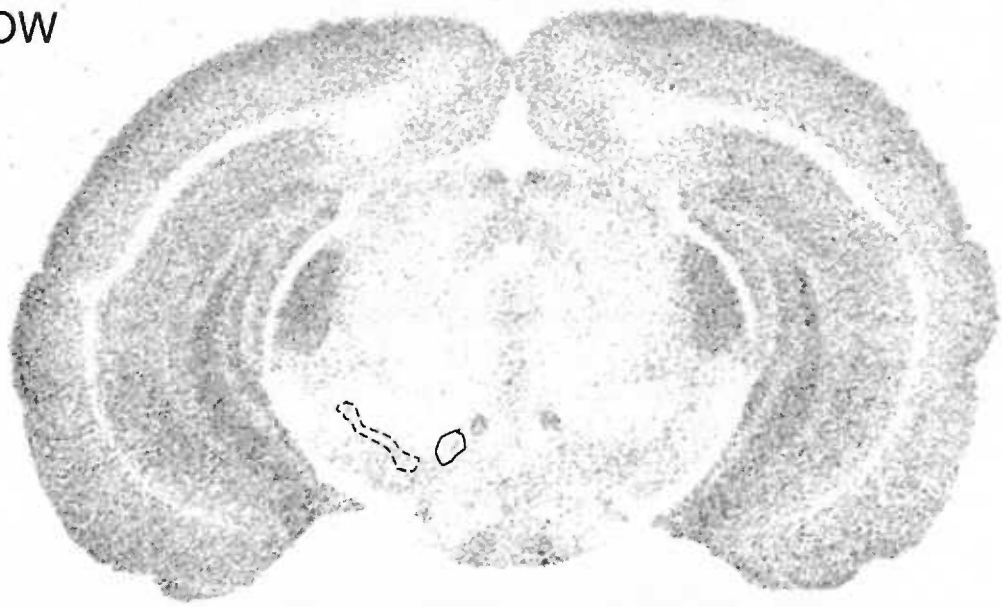
B.

-3.08 mm

FAST

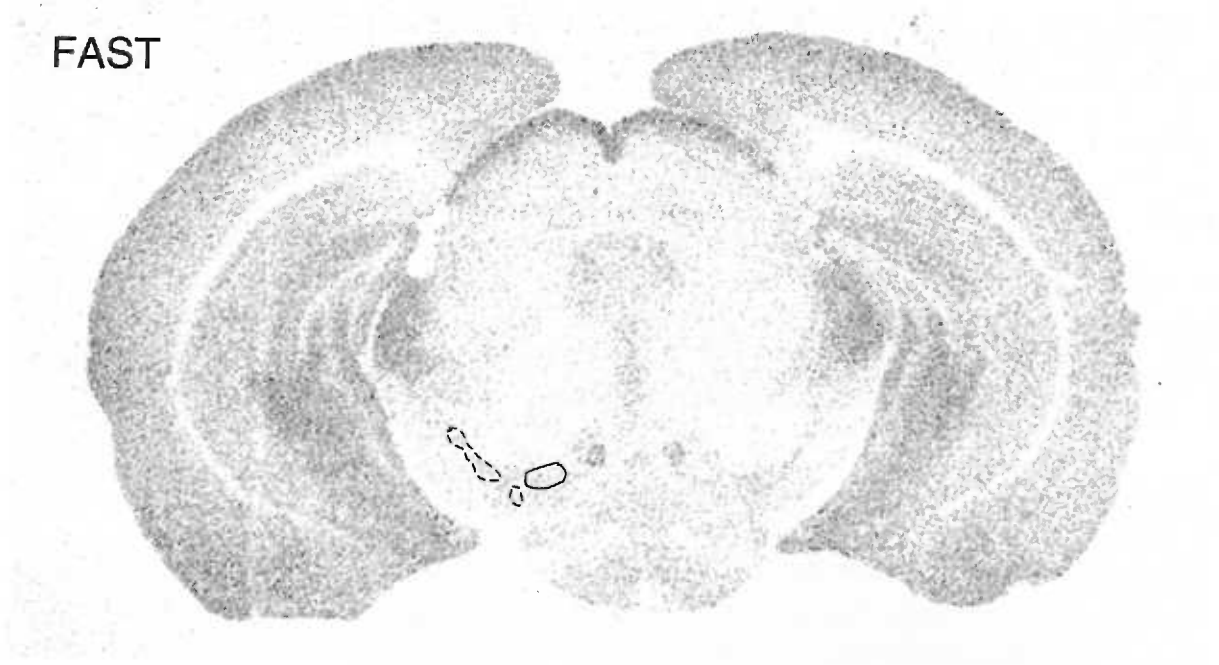


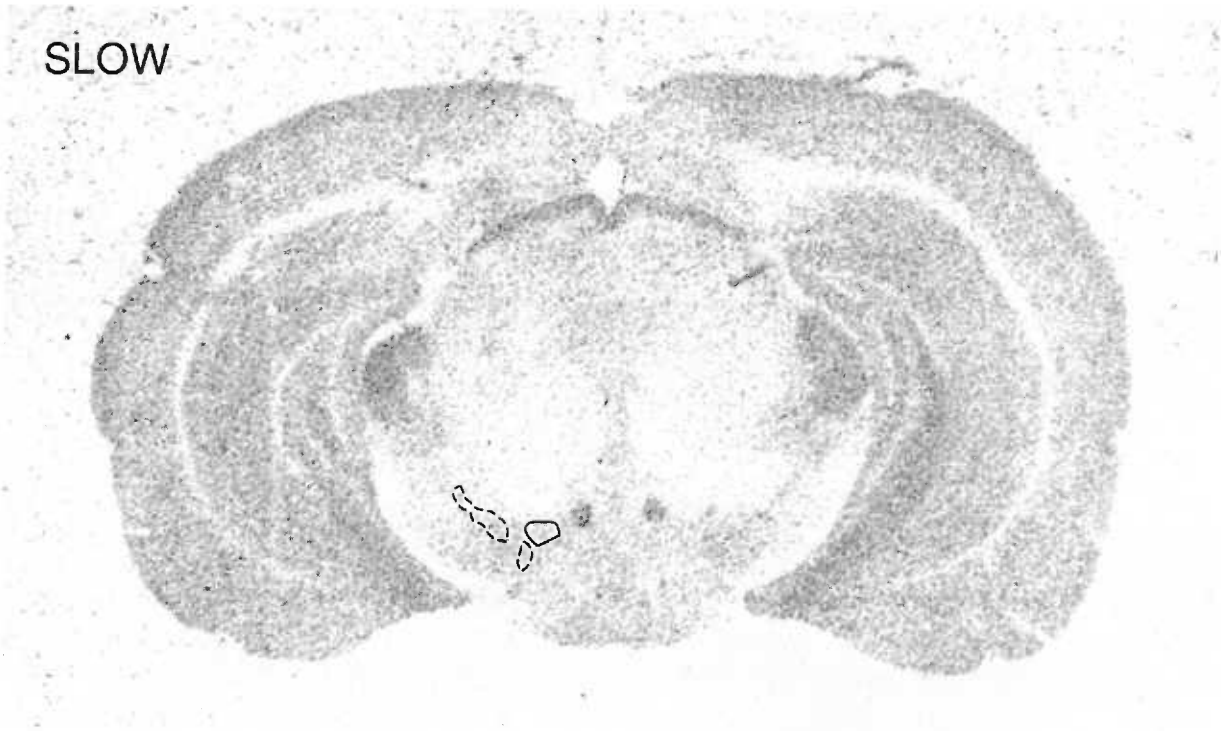
SLOW



C.

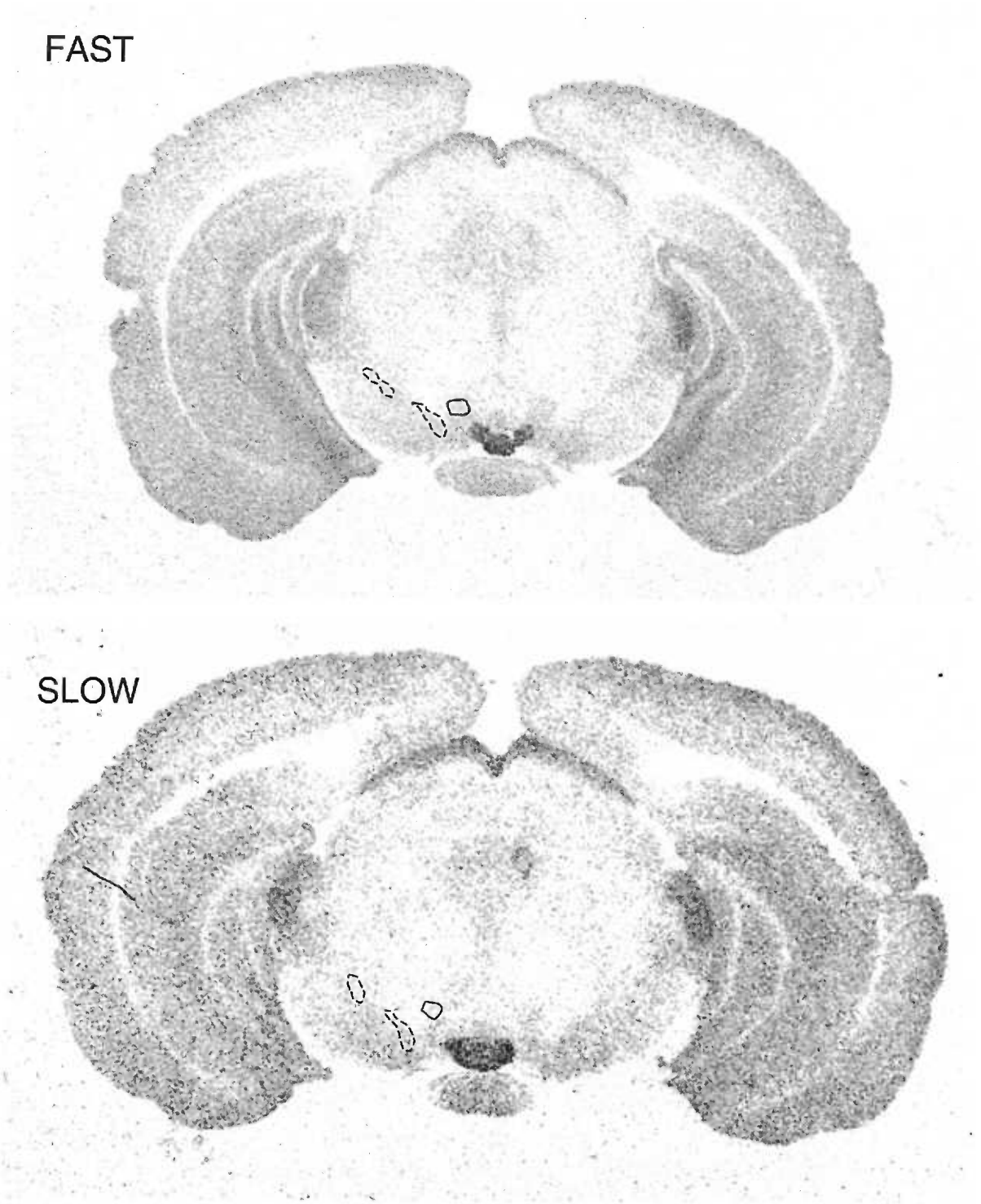
-3.16 mm





D.

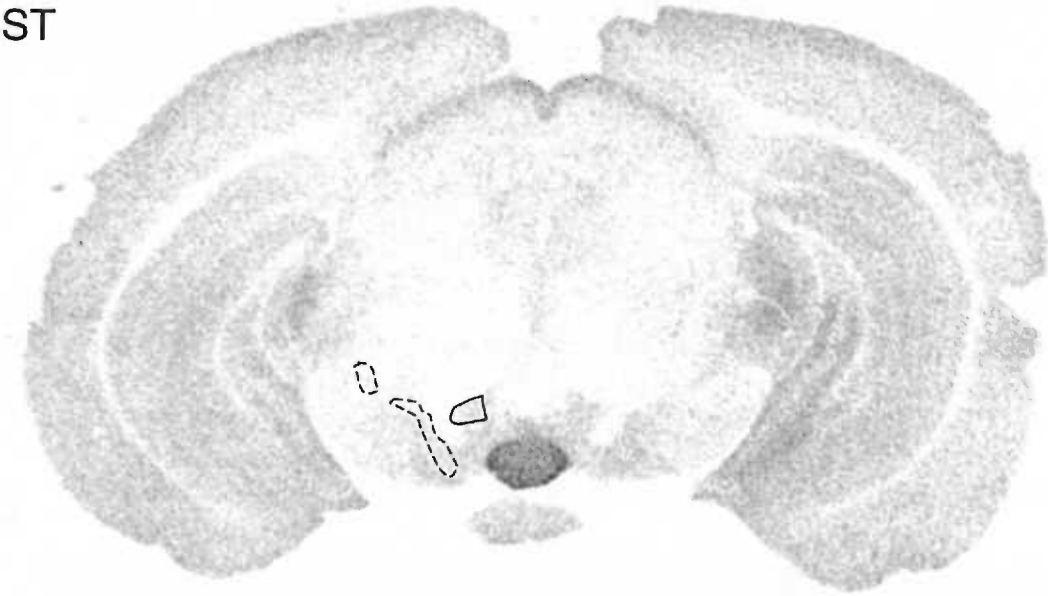
-3.28 mm



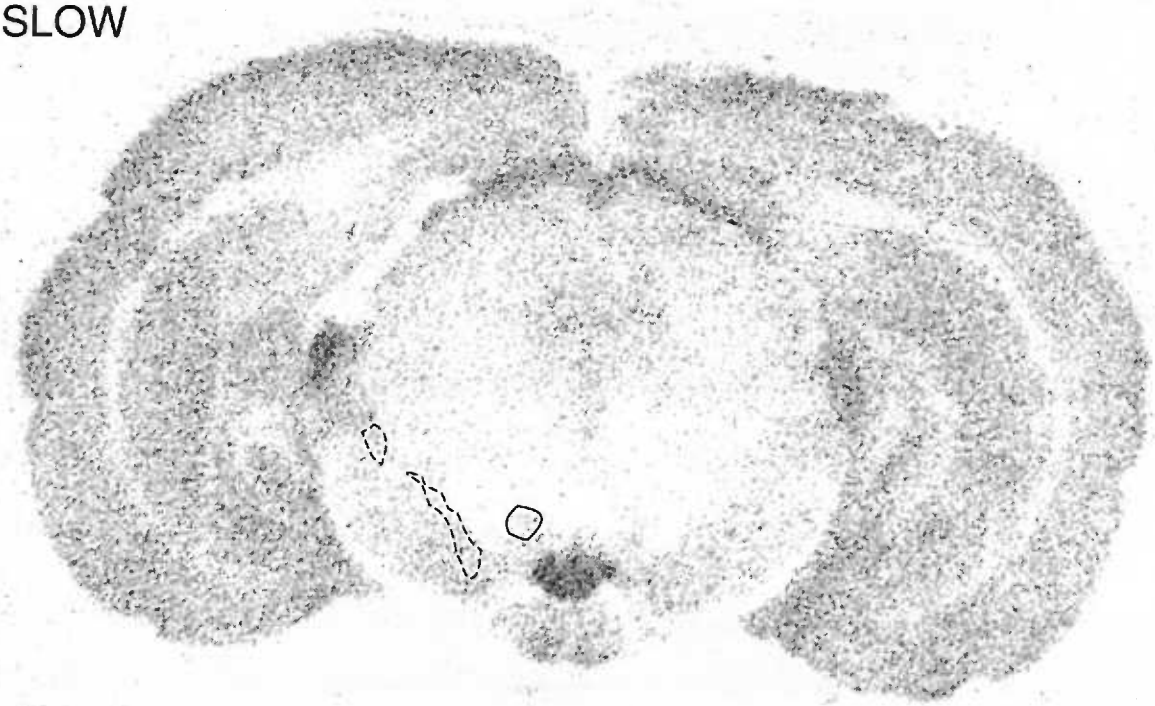
E.

-3.40 mm

FAST



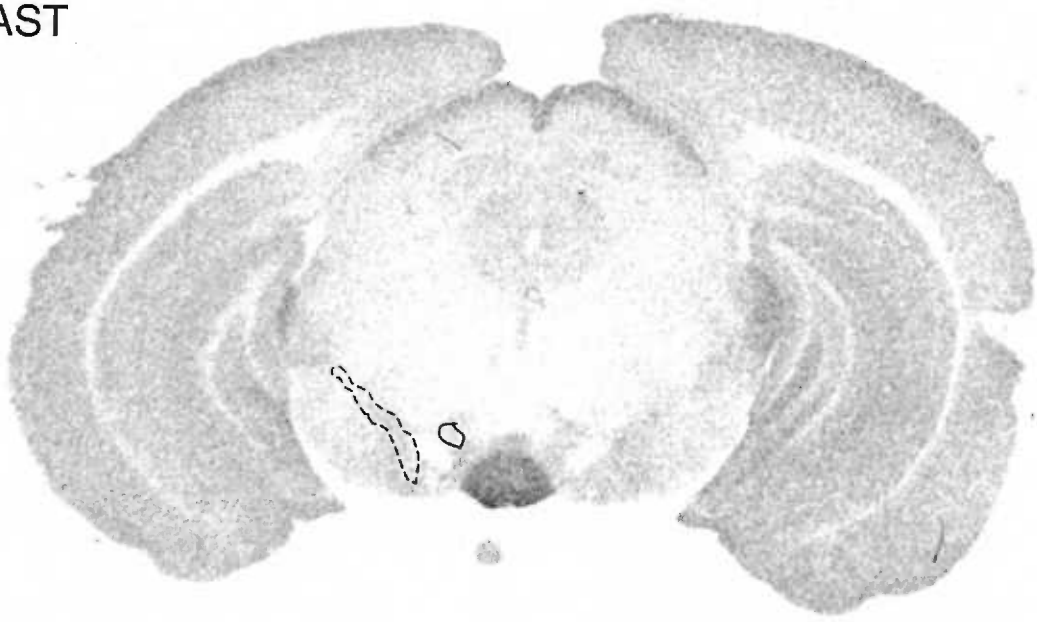
SLOW



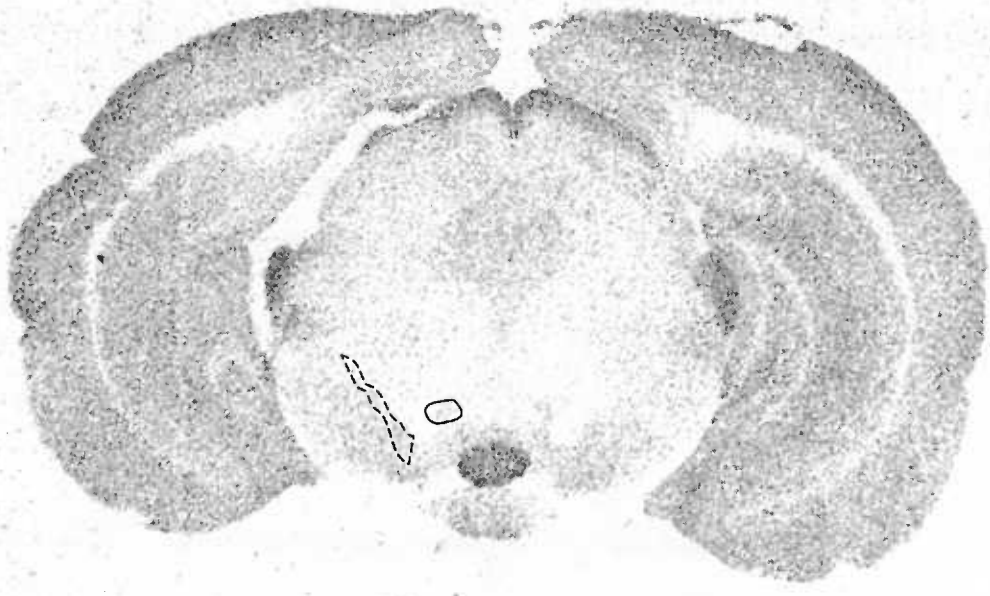
F.

-3.52 mm

FAST

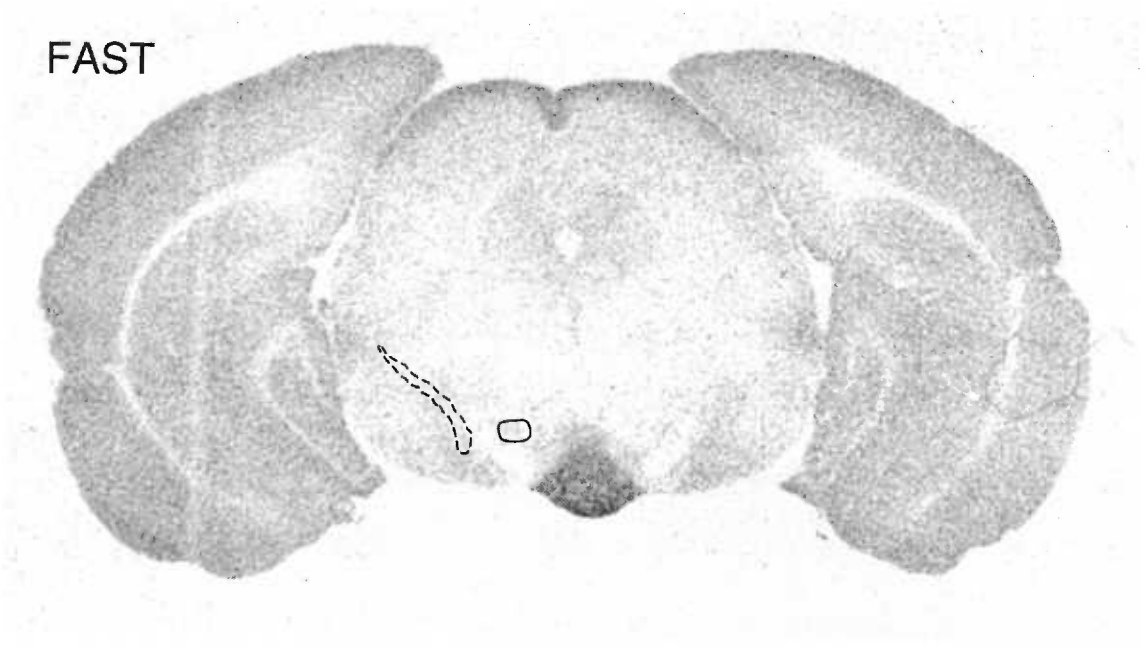


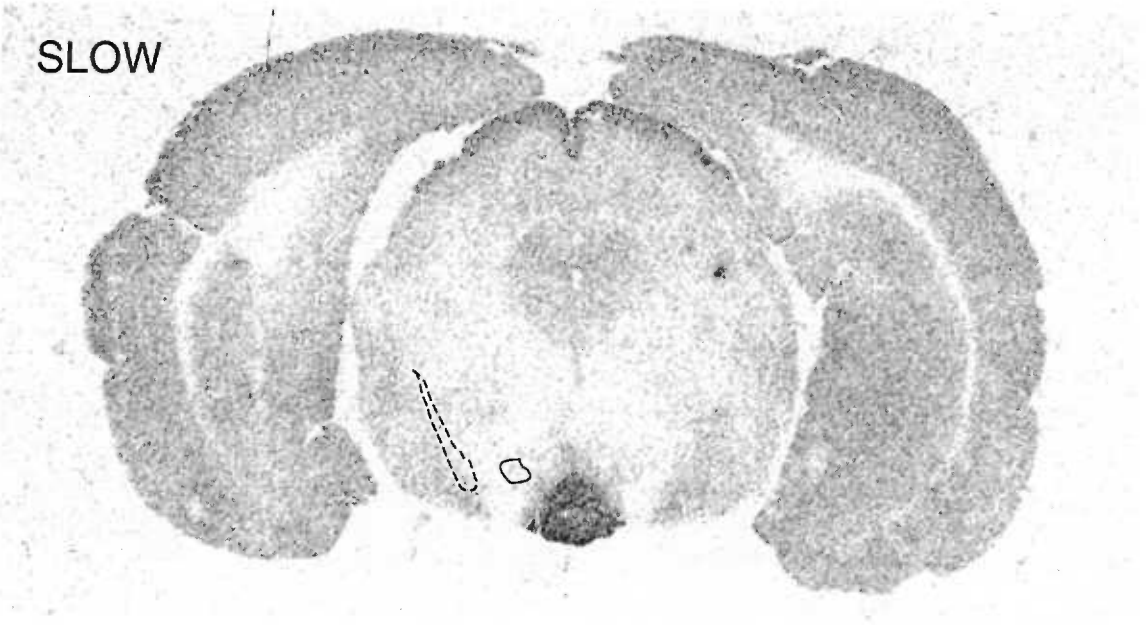
SLOW



G.

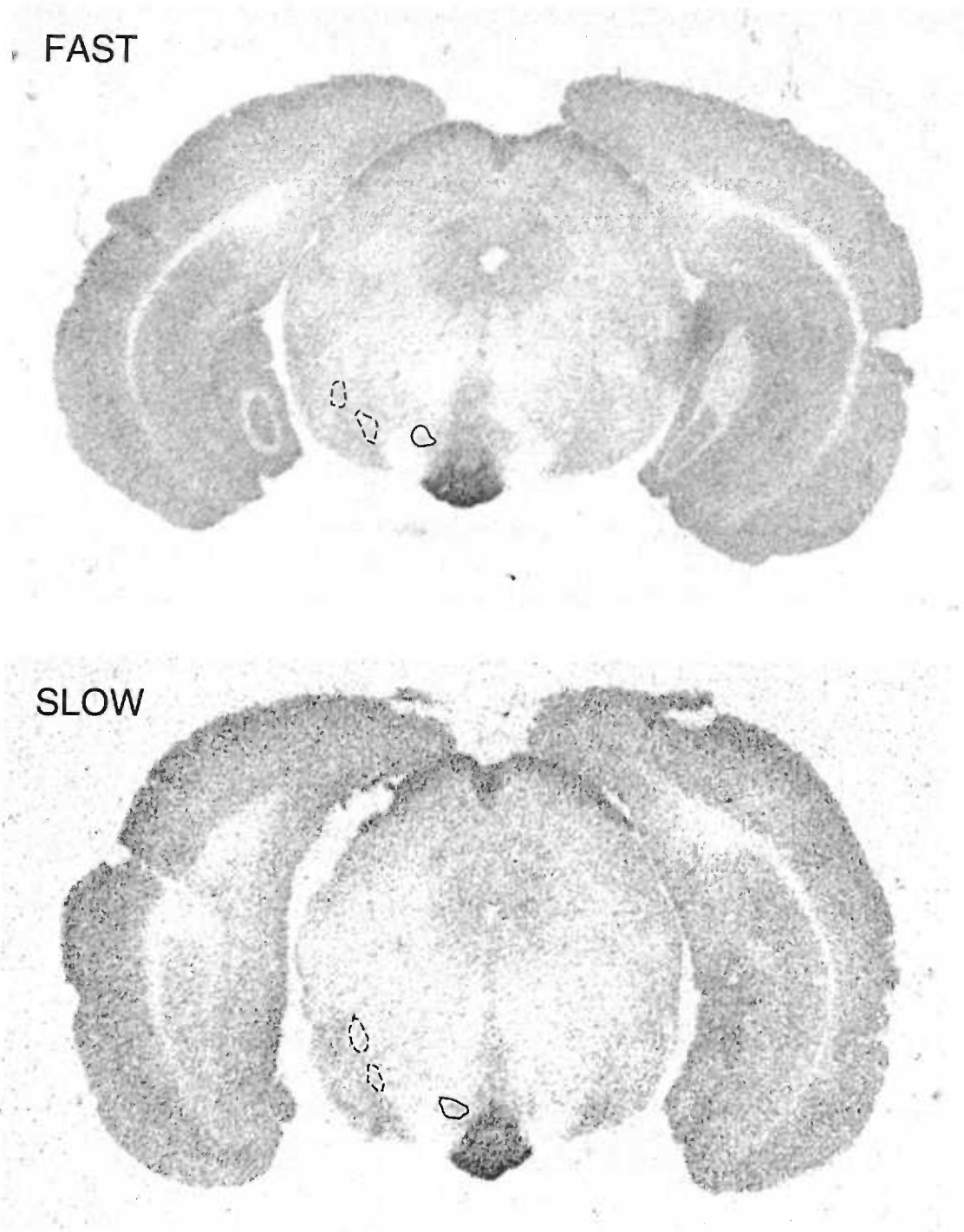
-3.64 mm





H.

-3.80 mm



I.

-3.88 mm

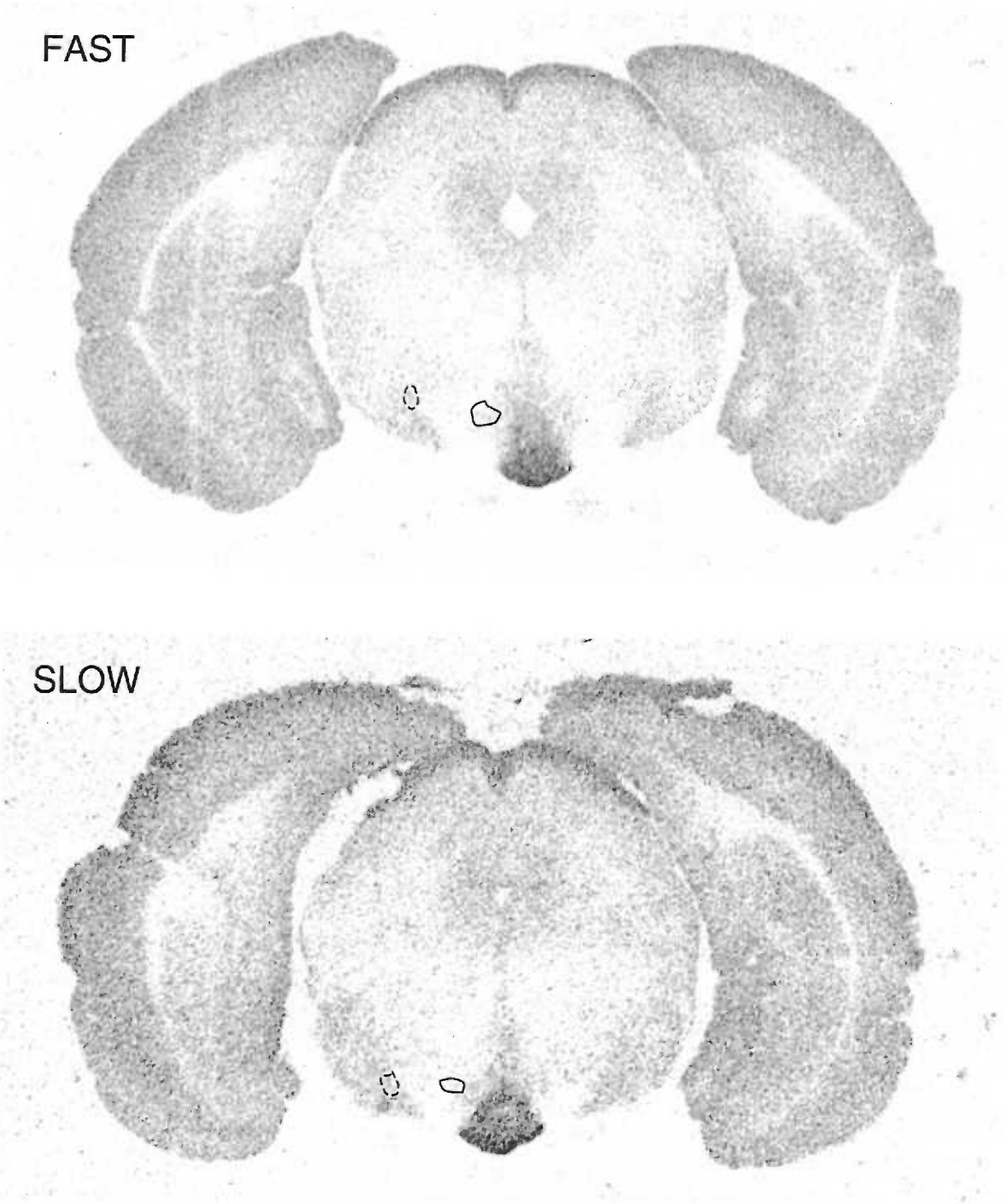
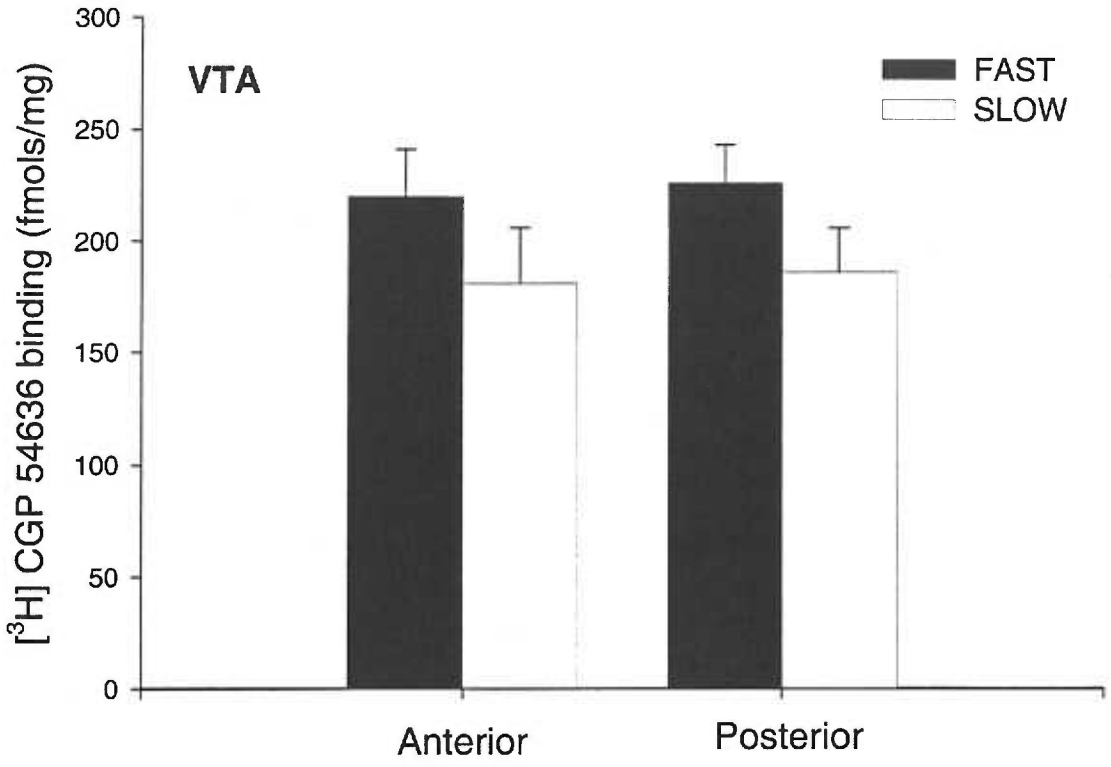
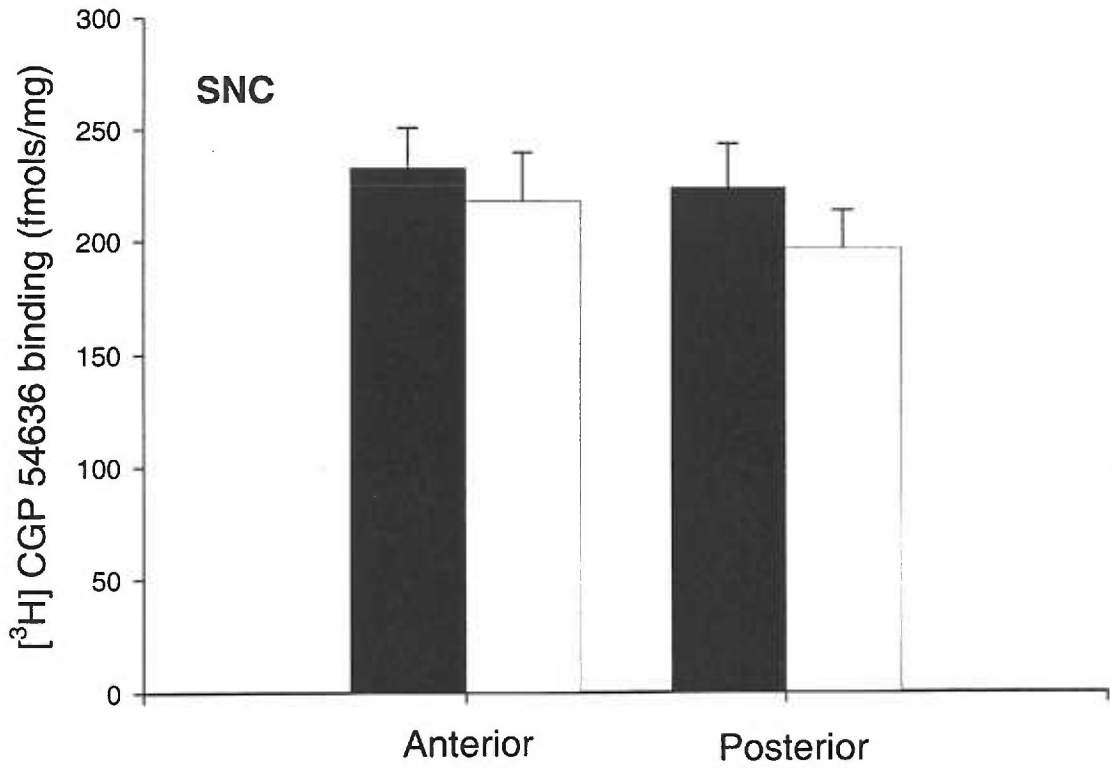


Figure 10. $GABA_B$ receptor densities in the anterior and posterior regions of the ventral tegmental area (upper panel) and substantia nigra compacta (lower panel) of FAST and SLOW mice. [^{3}H]CGP 54626 binding densities were summed, and then averaged for the anterior (-2.92 to –3.4 mm, with respect to bregma) and posterior (-3.52 to –3.88 mm, with respect to bregma) regions of the two structures in each mouse. $GABA_B$ receptor densities are expressed in fmols/mg tissue. Neither anterior, nor posterior VTA or SNC $GABA_B$ receptor densities differed between the FAST and SLOW lines.


VTA
FAST
SLOW
[3H] CGP 54636 binding (fmols/mg)
300
250
200
150
100
50
0
Anterior
Posterior


SNC
[3H] CGP 54636 binding (fmols/mg)
300
250
200
150
100
50
0
Anterior
Posterior

over the anterior (-2.92 to -3.4 mm, with respect to bregma) and posterior (-3.52 to -3.88 mm, with respect to bregma) regions of the VTA, and analyzed by separate one-way ANOVAs, no line differences were detected (F's[1, 11] = 1.37-2.19, p's = 0.17-0.27). Thus, FAST and SLOW mice do not appear to differ in $GABA_B$ receptor density in either VTA region (upper panel, Figure 10).

Substantia Nigra Pars Compacta (SNC). The SNC is a neural structure located just ventrolateral to the VTA that is thought to have a role in general locomotion. Baclofen may have diffused to the SNC and produced the behavioral effects described in Chapter 1. As seen in Table 2 and Figure 9, the pattern of $GABA_B$ receptor binding also varied anterior to posterior through the SNC. When $GABA_B$ receptor densities were summed and averaged across the entire SNC (-2.92 to -3.88 mm, with respect to bregma), one-way ANOVA did not reveal a significant difference between the lines (229±16 and 209±15 fmols/mg tissue for FAST and SLOW mice, respectively; F[1, 11) = 0.73, p = 0.41). Moreover, when the anterior and posterior regions of the SNC (calculated as described above) were analyzed separately, no significant line differences in $GABA_B$ receptor densities were found (F[1, 11] = 0.27-0.96, p's = 0.35-0.62). Thus, similar to $GABA_B$ receptor densities measured in the VTA, anterior and posterior $GABA_B$ receptor densities in the SNC did not appear to differ significantly between the lines (lower panel, Figure 10).

Discussion

Shen et al. (1998) showed that systemic baclofen administration produced a greater locomotor sedative effect in SLOW-1 mice compared to FAST-1 mice, and in

Chapter 2 we described data in which anterior intra-VTA microinjection of baclofen produced a locomotor sedative effect in SLOW, but not FAST mice. Several possibilities may explain these results. In the current study, we tested the possibility that genetic selection for differential sensitivity to EtOH's locomotor stimulant effects may have resulted in a greater $GABA_B$ receptor density in the anterior region of the VTA in SLOW mice. However, whether estimated for the entire structure, or for the anterior and posterior regions, $GABA_B$ receptor autoradiography using [^{3}H]CGP 54626 failed to detect differences in VTA $GABA_B$ receptor density between the FAST and SLOW lines.

Visual examination of Figure 8 suggested a trend toward greater $GABA_B$ receptor density in FAST, relative to SLOW mice. Based on the relatively small group sizes, and the appearance of this trend, we conducted a power analysis using the means and error associated with the current data set to determine the number of animals that would be needed to detect a significant difference between the lines, if one actually existed. This analysis suggested that we would need about 40 animals per group to achieve statistical power of 0.8 (Keppel, 1991). That is, given our small effect size, assessment of $GABA_B$ receptor densities in 40 animals per line would be necessary to correctly reject the null hypothesis of no line difference 80% of the time, if the alternative hypothesis was indeed true. Thus, we accept our finding that the lines do not differ in $GABA_B$ receptor density in either region of the VTA. However, if a difference does exist, it is modest at best and opposite to the direction of our hypothesis, and probably cannot fully account for the differential behavioral effects of baclofen seen between FAST and SLOW mice.

Whereas the above results do not support a line difference in $GABA_B$ receptor densities, they do not rule out the possibility that there are differences in $GABA_B$ receptor

subunit composition in the VTA of FAST and SLOW mice. Differences in receptor composition could result in differences in receptor function. Recently, two different splice variants of the $GABA_B$ receptor, the $GABA_{B1a}$ and $GABA_{B1b}$ splice variants, were identified (Kaupmann et al, 1997). Furthermore, several groups recently discovered a second $GABA_B$ receptor subunit, $GABA_{B2}$ (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999). Current data suggest that functional $GABA_B$ receptors form a heterodimer consisting of one of the $GABA_{B1}$ receptor splice variants, and the $GABA_{B2}$ receptor subunit, however, functional homodimers have not been conclusively ruled out (Kaupmann et al., 1998). Thus, another possible explanation for baclofen's behavioral effects may be that FAST and SLOW mice differ in $GABA_B$ receptor subunit composition of the anterior VTA. This possibility is intriguing since the distributions of these receptor splice variants differ markedly throughout the rat brain (Bischoff et al., 1999). Futhermore, [^{3}H]CGP 54626 has been shown to bind both the $GABA_{B(1a, 2)}$ and $GABA_{B(1b, 2)}$ receptor heterodimers, although with slightly higher affinity for $GABA_{B(1b, 2)}$ (1.51 versus 0.86 nM; Kaupmann et al., 1998). Given that we performed our assay using 2 nM [^{3}H]CGP 54626, it is possible that we saturated $GABA_{B(1b, 2)}$, but not $GABA_{B(1a, 2)}$, perhaps resulting in preferential binding to $GABA_{B(1b, 2)}$. Regardless, *in situ* hybridization studies, or possibly $GABA_B$ receptor autoradiography using a range of [^{3}H]CGP 54626 concentrations, will be necessary to assess potential differences in $GABA_B$ receptor subunit composition between the FAST and SLOW lines.

That $GABA_B$ receptor subunit compositions could possibly differ between the FAST and SLOW lines begs the question of whether $GABA_B$ receptor function has been

altered. Any alteration in the functional competence of $GABA_B$ receptors between the FAST and SLOW lines could be the direct result of a difference in receptor subunit composition, or could indirectly result from an alteration in one or several of the many $GABA_B$ receptor effector systems. Agonist binding to $GABA_B$ receptors results in stimulation of $Gi\alpha$, which in turn inhibits adenylate cyclase activity (Wojcik and Neff, 1984; Hill, 1985; Karbon and Enna, 1985; Andrade et al., 1986). $GABA_B$ receptor agonist binding also results in an opening of presynaptic calcium channels, and postynaptic inwardly rectifying potassium channels (Cherubini and North, 1984; Deisz and Lux, 1985; Inoue et al., 1985a; Inoue et al., 1985b). Moreover, whereas alone $GABA_B$ receptor activation is known to inhibit adenylate cyclase activity, evidence suggests that along with activation of $Gs\alpha$-coupled metabotropic receptors, $GABA_B$ receptor activation can facilitate adenylate cyclase induction, suggesting that $GABA_B$ receptors influence other neuromodulatory systems (Karbon and Enna, 1985). Thus, genetic selection for extreme sensitivities to EtOH-stimulated locomotor activity may lead to differential sensitivities to agonist-induced inhibition of adenylate cyclase, altered enhancement of calcium or potassium flux, and/or altered function of another neuromodulatory system that is influenced by $GABA_B$ receptors. In other words, SLOW mice might be more sensitive to the locomotor sedative effects of anterior intra-VTA baclofen because $GABA_B$ receptors located on the DA neurons in this VTA region may stimulate adenylate cyclase, enhance potassium influx, and/or alter the function of another neural system that the $GABA_B$ system interacts with. Future studies will utilize functional assays in FAST and SLOW mice to address these possibilities.

The SNC is a structure located just ventrolateral to the VTA, and is known to have a role in mediating general locomotion. Autoradiography and in situ hybridization studies in rats suggest the presence of $GABA_B$ receptors in the SNC (Bowery et al., 1987; Chu et al., 1990; Bischoff et al., 1999; Lu et al., 1999). Consequently, we reasoned that in our behavioral study (see Chapter 1), baclofen may have diffused to this structure to produce its differential effects on locomotion in FAST and SLOW mice. Thus, we also assessed the binding density pattern of [^{3}H]CGP 54626 in the SNC of FAST and SLOW mice. Similar to the results seen in the VTA, analysis of data averaged over the entire SNC did not reveal a significant line effect, nor did similar separate analyses for the anterior and posterior regions of the SNC. These results do not support a role for different numbers of $GABA_B$ receptors in the SNC of FAST and SLOW mice in mediating baclofen's behavioral effects in our microinjection study. However, similar to the alternative possibilities put forth for the VTA, FAST and SLOW mice may differ in the subunit composition and thus function of $GABA_B$ receptors in the SNC or in other circuits influenced by the SNC. Future studies will be necessary to address these possibilities.

In conclusion, we have shown that $GABA_B$ receptor densities in the anterior and posterior regions of the VTA do not differ between the FAST and SLOW selected mouse lines. Moreover, the lines also do not differ in $GABA_B$ receptor densities in the anterior and posterior regions of the SNC. Thus, the current data do not support the hypothesis that $GABA_B$ receptor densities in the anterior region of the VTA were differentially altered by genetic selection for differential sensitivity to EtOH's locomotor stimulant effects. Future studies will examine whether the enhanced sensitivity of SLOW mice to

the locomotor sedative effects of anterior intra-VTA baclofen are due to a selection-induced line difference in VTA $GABA_B$ receptor subunit composition, function, and/or another neurotransmitter system that is influenced by $GABA_B$ receptor activation.

OVERALL DISCUSSION

Based on localization data, it seemed plausible that baclofen's antagonism of EtOH-stimulated activity in a previous study in FAST and SLOW mice (Shen et al., 1998) was mediated by central $GABA_B$ receptors in the VTA. ICV baclofen administration demonstrated that agonist binding to central $GABA_B$ receptors could dose-dependently attenuate EtOH's locomotor stimulant effects in FAST mice (Boehm II et al., *submitted*). My dissertation project showed that $GABA_B$ receptors in the VTA can specifically mediate this effect. Intra-VTA injection of baclofen attenuated EtOH-stimulated locomotion in FAST mice. However, baclofen microinjected into the posterior region of the VTA had the opposite effect; it *enhanced* EtOH's locomotor stimulant effects.

The opposite effect of posterior intra-VTA microinjection of baclofen on EtOH-stimulated locomotor activity in FAST mice is consistent with a number of studies that have suggested regional heterogeneity with regard to organization of GABA systems within the VTA (Arnt and Scheel-Kruger, 1979; Wirtshafter and Klitenick MA, 1989; Ikemoto et al., 1997; 1998). Our locomotor stimulant results parallel those for VTA region-specific EtOH self-administration (Rodd-Henricks et al., 2000b). The current data support the notion that portions of the VTA are part of a neural circuit mediating both the stimulant and rewarding effects of EtOH. FAST mice have previously been found to voluntarily self-administer relatively more EtOH than SLOW mice (Risinger et al., 1994). Our data suggest that FAST mice would readily self-administer EtOH to the posterior VTA, but not the anterior VTA.

SLOW mice were included in our microinjection experiment when preliminary data suggested the potentiating effect of posterior intra-VTA microinjection of baclofen in FAST mice. However, neither posterior, nor anterior, intra-VTA microinjection of baclofen altered EtOH's effects on locomotion in these mice. When baclofen was microinjected into the anterior region of the VTA, it reduced locomotion, to a greater extent in SLOW than in FAST mice, a result that is consistent with that reported previously in which SLOW-1 mice were more sensitive than FAST-1 mice to the locomotor sedative effects of systemically administered baclofen (Shen et al., 1998). Thus, we speculated that selection for increased or decreased sensitivity to EtOH stimulation may have differentially altered anterior VTA $GABA_B$ receptors such that SLOW mice exhibit a greater receptor density and/or receptor function than FAST mice.

In Chapter 2, we addressed the possibility that compared to FAST mice, the anterior VTA of SLOW mice possesses a greater density of $GABA_B$ receptors, making this line more sensitive to baclofen. The results of our autoradiographic analysis did not support our hypothesis; the FAST and SLOW lines did not significantly differ in $GABA_B$ receptor densities in the anterior or posterior regions of the VTA. However, it is now known that there at least two $GABA_B$ receptor subunits (in addition to splice variants of one of the subunits), and that these components interact in determining receptor function (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1998). Thus, techniques such as *in situ* hybridization will be necessary to elucidate whether the line differences in baclofen sensitivity can be attributed to differing $GABA_B$ receptor subunit composition.

If the FAST and SLOW lines indeed differ in $GABA_B$ receptor subunit composition, a result could be differential receptor function. In addition to possible differences at the level of $Gi\alpha$ activation and thus inhibition of adenylate cyclase (Wojcik and Neff, 1984; Hill, 1985; Karbon and Enna, 1985; Andrade et al., 1986), and changes in presynaptic calcium channels or postynaptic inwardly rectifying potassium channels (Cherubini and North, 1984; Deisz and Lux, 1985; Inoue et al., 1985a; Inoue et al., 1985b), another potential explanation for the current results concerns $GABA_B$ receptor interactions with other neurotransmitter systems relevant to VTA function. In an early study, Karbon and Enna (1985) investigated the effects of baclofen treatment on cyclic AMP accumulation in cortical slices. As discussed above, $GABA_B$ receptors are known to inhibit adenylate cyclase (Wojcik and Neff, 1984; Hill, 1985; Karbon and Enna, 1985; Andrade et al., 1986). However, when baclofen was co-administered with histamine, isoproterenol, norepinephrine, vasoactive intestinal peptide, adenosine, or 2-chloroadenosine, cyclic AMP accumulation increased by 2-3 fold (Karbon and Enna, 1985). Considered along with several other reports, it was concluded that when $GABA_B$ receptors are activated along with G-protein coupled receptors that stimulate adenylate cyclase ($Gs\alpha$), $GABA_B$ receptor activation can actually *enhance* stimulation of adenylate cyclase (Wojcik and Neff, 1984; Hill, 1985; Karbon and Enna, 1985). Termed coincident signaling, a model has been proposed that explains these effects in terms of $G\beta\gamma$ liberation (Cunningham and Enna, 1997). Activation of stimulatory G-protein coupled receptors associated with $Gs\alpha$ partially stimulates adenylate cyclase types II and IV. However, activation of other metabotropic G-protein coupled receptors that liberate $G\beta\gamma$, such as $GABA_B$ receptors, potentiate this stimulation, resulting in enhanced production of

cyclic AMP. Thus, these data provide evidence that $GABA_B$ receptors interact with other neurotransmitter signaling pathways, suggesting another explanation for the greater sensitivity of SLOW mice to anterior intra-VTA microinjection of baclofen; genetic selection may have altered the function of another neurotransmitter system that interacts with $GABA_B$ receptor signaling.

In as much as the structural organization of the VTA may influence its function, one might also be able to explain our behavioral data in terms of dopamine cell density. Data suggest that the concentration of dopamine cell bodies drops sharply in more posterior regions of the VTA, and that the number and distribution of dopamine cells within the VTA is genotype dependent with FVB/N inbred mice possessing a larger population of dopamine cell bodies in the VTA compared to C57BL/6 inbred mice (Nelson et al., 1996). Interestingly, these strains appear to differ in sensitivity to EtOH's locomotor stimulant effects; FVB/N mice exhibit a greater sensitivity to this EtOH effect (John Crabbe, personal communication). Together, these data suggest the possibility that the FAST and SLOW lines could differ in dopamine cell number, distribution, or both. FAST mice could possess a greater density of dopamine neurons in the anterior VTA, making them more sensitive to the locomotor stimulant effects of EtOH. Considered along with our $GABA_B$ receptor autoradiographic data showing no line differences in receptor densities in either the anterior or posterior VTA, this scenario could also explain why FAST mice are less sensitive to anterior intra-VTA baclofen. Compared to SLOW mice, FAST mice might express fewer $GABA_B$ receptors relative to the number of dopamine neurons in the anterior VTA, resulting in reduced sensitivity to intra-VTA baclofen.

$GABA_B$ receptors have been localized to dopamine (DA) cell bodies in the VTA. Thus baclofen my directly inhibit EtOH's stimulation of DA release, and presumably, EtOH's locomotor stimulant effects. A cartoon illustrating this idea can be seen in Figure 11. However, this scheme cannot explain the posterior effects of intra-VTA baclofen seen in ethanol-treated FAST mice. A number of possibilities may explain these results. In the simplest of these, posterior VTA $GABA_B$ receptors might be localized on GABA interneurons that synapse on DA cell bodies (see Figure 12), resulting in an inhibition of GABA interneurons in the presence of baclofen, and resultant disinhibition of DA neurons. Alternatively, similar disinhibitory effects would be seen if $GABA_B$ receptors in the posterior VTA were localized on GABA neurons that, in addition to DA neurons, are also known to project to the NAcc (Van Bockstaele and Pickel, 1995; Laviolette et al., 2001). Our autoradiographic study confirmed the existence of $GABA_B$ receptors in both the anterior and posterior regions of the VTA in both FAST and SLOW mice. However, future studies will be needed, for example, utilizing immuno-labeling techniques to visualize $GABA_B$ receptors, as well as DA and GABA containing neurons, to elucidate differences in $GABA_B$ receptor localization between the anterior and posterior VTA of FAST and SLOW mice.

Given the region specific differences in intra-VTA baclofen seen in both the FAST and SLOW lines, it is clear that a detailed analysis of the structure and function of the anterior and posterior regions of the VTA is warranted. Help may be found in experiments incorporating electrophysiological techniques. A powerful procedure that may hold some promise involves single unit recordings from brain slice preparations. Using such techniques, for instance, one might be able measure the rate of VTA DA cell

Figure 11. Anterior VTA $GABA_B$ receptor localization. $GABA_B$ receptors are localized on the DA cell bodies in the VTA. $GABA_B$ receptor activation directly inhibits DA cell firing, reducing EtOH-induced DA release in the NAcc, and presumably EtOH-stimulated locomotion.

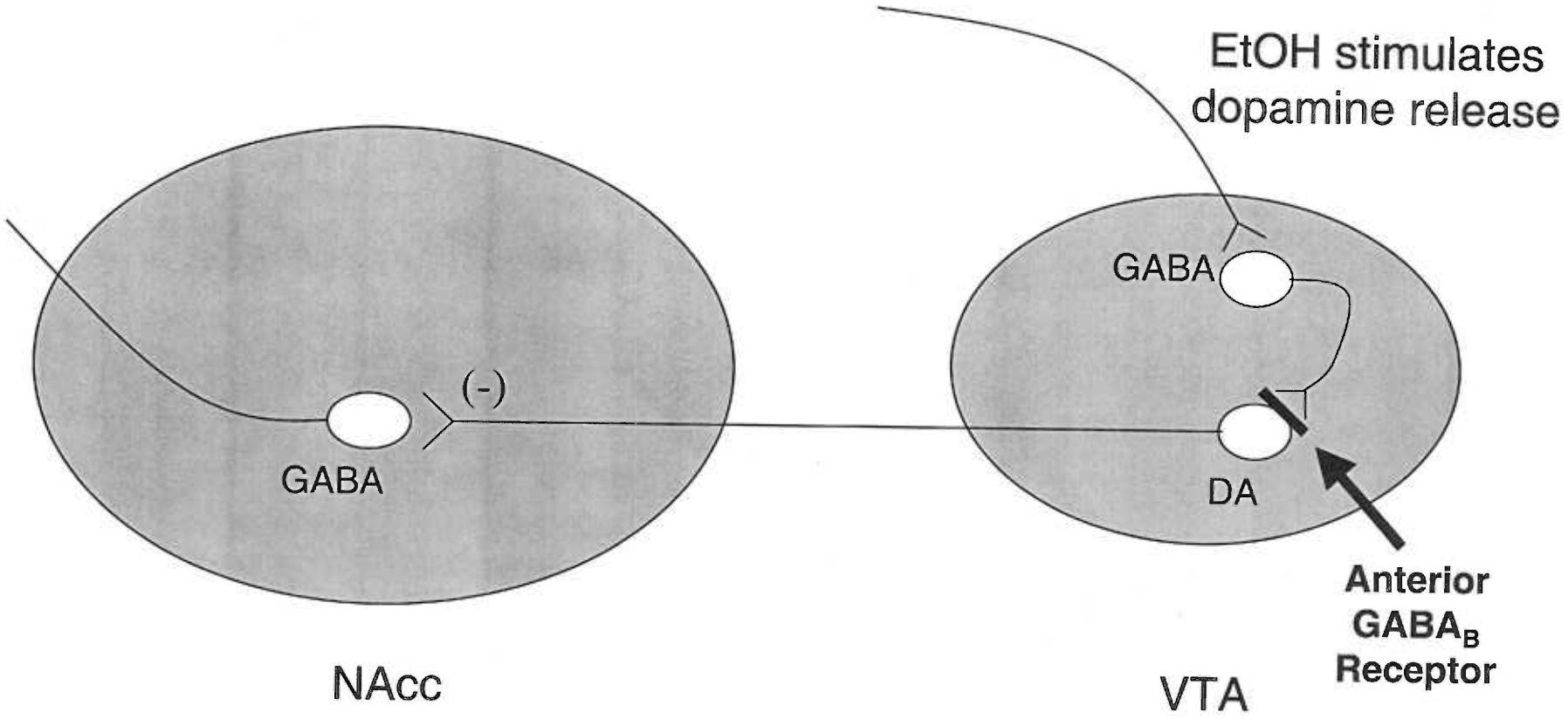
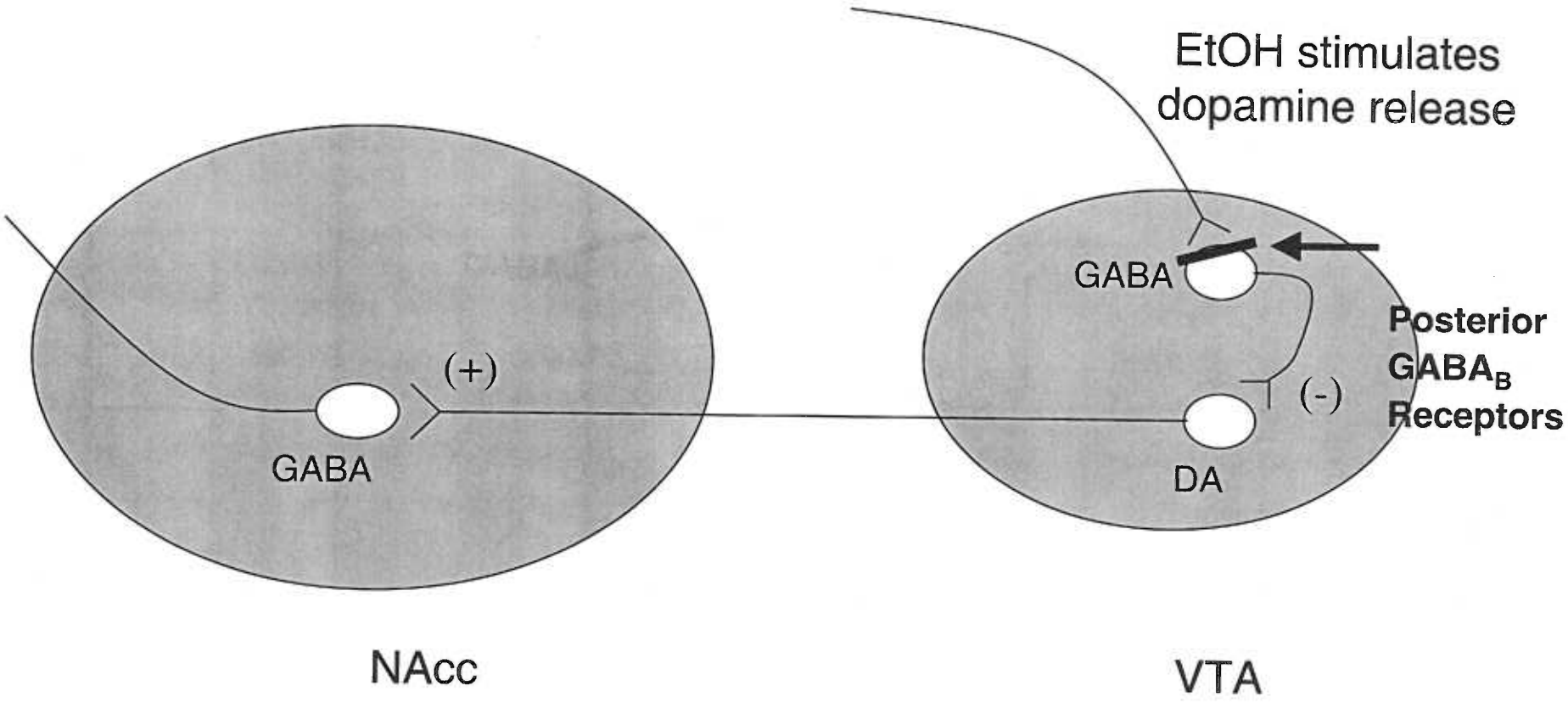

EtOH stimulates
dopamine release
GABA
(-)
GABA
DA
Anterior
GABA$_B$
Receptor
NAcc
VTA

Figure 12. Posterior VTA $GABA_B$ receptor localization. $GABA_B$ receptors are localized on GABA interneurons in the VTA. $GABA_B$ receptor activation directly inhibits the GABA interneurons resulting in a disinhibition of the DA neurons, reducing EtOH-induced DA release in the NAcc, and presumably EtOH-stimulated locomotion.


EtOH stimulates
dopamine release
GABA
Posterior
GABA$_B$
Receptors
(-)
(+)
GABA
DA
NAcc
VTA

firing in brain slices obtained from both FAST and SLOW mice, comparing the anterior and posterior regions. Moreover, these electrophysiological recordings could be obtained from brain slices both in the presence and absence of EtOH. Given the data of Brodie et al. (2000) suggesting a larger increase in the excitation of VTA DA neurons in mice that are more sensitive to the locomotor stimulant effects of EtOH (DBA/2J inbred strain) than are insensitive mice (C57BL/6J inbred strain), the prediction would be that EtOH would promote DA cell firing in FAST, but not SLOW mice. Furthermore, one might predict that the anterior and posterior regions of the VTA would respond differently to the presence of baclofen in the bath. In the absence of EtOH, the prediction would be that the DA neurons in the anterior VTA of SLOW mice would be more sensitive to baclofen (i.e., greater depression of DA cell firing) than those of FAST mice. Moreover, in the presence of EtOH, the anterior VTA DA cell bodies of FAST mice would exhibit reduced firing in response to baclofen, whereas the posterior DA cell bodies of FAST mice would exhibit increased firing in response to baclofen. Thus, considered along with immuno-labeling data, electrophysiological experiments such as these would strongly support or refute the notion that anterior VTA $GABA_B$ receptors are located on DA cell bodies, and that posterior $GABA_B$ receptors are located on GABA interneurons.

It is not immediately clear why posterior intra-VTA baclofen did not induce a locomotor stimulant response to EtOH in SLOW mice. Our autoradiographic results suggest it is unlikely that SLOW mice simply possess fewer $GABA_B$ receptors in the posterior, compared to anterior VTA. However, it may be that posterior $GABA_B$ receptors are less sensitive to baclofen than are those in the anterior VTA. Considered within the context of the above discussion on $GABA_B$ receptor localization in the two

VTA regions, posterior intra-VTA injection of baclofen might be expected to disinhibit the DA cell bodies in SLOW mice, resulting in an increase in DA cell firing, similar to that seen in FAST mice. However, if these $GABA_B$ receptors are less sensitive to agonist, higher baclofen doses might be necessary to influence downstream DA signaling.

In addition to having been selected for relative resistance to EtOH's locomotor stimulant effects, SLOW mice may also have been selected for enhanced sensitivity to EtOH-induced sedation. Often times during selection, breeders for subsequent generations of SLOW mice were chosen that exhibited slightly negative locomotor activity scores (i.e., EtOH activity scores that were lower than saline activity scores), perhaps increasing the frequency of alleles influencing enhanced sensitivity to EtOH's locomotor sedative effects in these lines. Indeed, ethanol-treated SLOW mice exhibit a longer duration of the loss of righting reflex than do similarly treated FAST mice (Shen et al., 1996; Phillips et al., *submitted*). This may also explain why SLOW mice did not stimulate to EtOH when baclofen was microinjected into the posterior region of the VTA. It is possible that in SLOW mice, EtOH's locomotor sedative effects completely mask EtOH's locomotor stimulant effects, and that posterior intra-VTA baclofen is not sufficient to overcome them. Thus, future studies might utilize lower EtOH doses to reduce locomotor sedation in the SLOW lines. In this way, any potential effects of posterior intra-VTA baclofen in EtOH-treated SLOW mice might be revealed.

At first it may appear difficult to reconcile the current intra-VTA microinjection data with the earlier systemic and ICV data. Systemic and ICV administration of baclofen both resulted in a dose-dependent attenuation of EtOH's locomotor stimulant

effects (Shen et al., 1998; Boehm II et al., *submitted*), however, the current data suggests that intra-VTA baclofen can either attenuate or potentiate EtOH-stimulated activity depending on region of injection. However, the anterior intra-VTA effects of baclofen may overshadow its posterior intra-VTA effects. In other words, $GABA_B$ receptors in the anterior region of the VTA may ultimately mediate the locomotor output of the system, even when posterior $GABA_B$ receptors are also activated. Indeed, the current data showing that SLOW mice are more sensitive to anterior intra-VTA effects of baclofen also support a dominant role for anterior VTA $GABA_B$ receptors. SLOW mice are insensitive to EtOH-stimulated locomotor activity, suggesting that $GABA_B$ receptor activation in this region of the VTA renders the DA cell bodies incapable of increased firing in response to EtOH.

In conclusion, we have shown that $GABA_B$ receptors in the anterior VTA can mediate baclofen's attenuating effects on EtOH-stimulated activity, but that general differences in sensitivity to the drug cannot be attributed to $GABA_B$ receptor density in the VTA. Moreover, regional heterogeneity of the VTA, similar to that seen for EtOH self-administration in rats, also generalizes to EtOH's locomotor stimulant effects in mice. Taken together, these data suggest that the anterior and posterior regions of the VTA may be structurally different, leading to differing behavioral outcomes when either region is manipulated relative to the other. Our data suggest an important $GABA_B$ influence on basal and EtOH-stimulated locomotion in the FAST and SLOW selectively bred mouse lines. However, future studies will be necessary to elucidate whether differences exist between FAST and SLOW mice in the neurocircuitry, neurochemistry, and neurophysiology associated with $GABA_B$ receptor activation. Finally, the ability to

isolate region-specific effects within the mouse VTA suggests an exciting future for research using this genetically rich species.

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