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ALCOHOL AND ANESTHETIC MECHANISMS IN GENETICALLY ENGINEERED MICE

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1. ABSTRACT

Genetically engineered animals (e.g., transgenics, gene knockouts, gene knockins) are being utilized with increasing frequency to investigate the mechanisms of action of alcohol and anesthetics. By creating and analyzing animals that harbor precise, preplanned changes in candidate genes, researchers are rapidly making progress toward uncovering how these drugs exert their effects on the central nervous system to bring about their behavioral effects. Since these sedative / hypnotic agents are likely to exert their effects by altering neurotransmission, the majority of investigations to date have focused on neurotransmitter receptors and modulators of neurotransmission such as kinases.

2. INTRODUCTION

General anesthetics are arguably society's most therapeutically useful drugs, while alcohol is certainly its most abused. Ironically, a century of research suggests a common mechanistic basis for their actions on the nervous system. Both general anesthesia and alcohol intoxication are disturbances of higher order nervous function responsible for complex behavior including consciousness. By definition, therefore, despite the enormous utility of *in vitro* work, true mechanistic insight into these processes can only be derived from observations in living, behaving animals. As early as 1899, Hans Meyer employed tadpoles to derive the durable correlation between an agents obtunding potency and its lipid solubility (1). Remarkably, the correlation was shown to prevail in rodents, primates, and people (2).

Physiological studies in rodents have been extremely useful in demonstrating critical portions of the nervous system's response to anesthetics. For example, decerebration does not change anesthetic potency in the rat (3). This suggests that the immobilizing effects of anesthetics are mediated by subcortical structures. Rats have also been used to demonstrate that stereotactic injection of the alpha 2 adrenergic agonist, dexmedetomidine, into the locus coeruleus produces a hypnotic response (4).

Classic genetic approaches in rodents to understanding mechanisms of intoxication and anesthesia have also been very informative (for reviews, see:5, 6). By selective breeding of rats or mice, numerous lines of animals have been developed that display divergent responses to various drugs including alcohol and anesthetics. Numerous inbred mouse strains and several spontaneously arising mutants have also been demonstrated to differ in response to alcohol and anesthetics. These traditional genetic studies, which began in the 1950's, have clearly established that the mechanisms which control various responses to drugs are under genetic control.

Recently, specific chromosomal regions which affect specific drug responses have been identified using the quantitative trait loci mapping technique. [For methodological overview, the reader is referred to a recent excellent review of this technique (7).] Chromosomal regions have been identified that control sensitivity to propofol (8) and various responses to alcohol (9-12). The next step in these studies is to identify the genes within these chromosomal regions that contribute to the drug responses. This technique holds great promise for identifying genes that form the pathways that control responses to alcohol and anesthetics.

The classic and quantitative genetics approaches are examples of forward genetics in which one makes an attempt to progress from an altered phenotype to the genetic basis for the observed differences. Despite several decades of considerable intense effort using the classic genetic approach, it has been nearly impossible to deduce the underlying genetic basis for the differential drug responses observed. To date, the only gene putatively identified in such animals has been the alpha 6 subunit of the $GABA_A$-R (13). The quantitative trait locus approach has yet to identify a gene that is directly responsible for an altered behavioral response to alcohol or anesthetics. It is expected that this very powerful approach will soon begin to yield insights into the molecular basis of action of these drugs.

A third genetic approach, reverse genetics (from gene to phenotype), provides a direct route for investigating the effect of specific candidate genes on the response to alcohol and anesthetics. With this approach, transgenic animals that express an additional gene, gene knockout animals that completely lack a single specific gene, or gene knockin animals that harbor a specific mutation in a predetermined gene are created and subsequently analyzed for altered drug responses. [The technical details of creating such animals are covered in numerous excellent reviews (14, 15) and thus will not be covered here.] Despite the fact that the reverse genetics approach has only recently been enlisted to investigate alcohol and anesthetic action, considerable mechanistic insight has already been gleaned from these studies and their results form the basis of this review.

3. NEUROTRANSMITTER RECEPTORS

3.1. Gamma-aminobutyric acid type A receptors

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. The type A GABA receptors ($GABA_A$-Rs) are the primary mediators of GABA induced rapid inhibitory neurotransmission (for review, see:16). $GABA_A$-Rs are heteropentameric membrane spanning protein complexes that conduct chloride ions into neurons in response to ligand binding. To date, 18 subunit polypeptides have been identified and these subunits can be grouped into families (alpha 1-6, beta 1-4, gamma 1-4, delta, epsilon, and rho 1-2) based on homology. The subunit composition of native $GABA_A$-Rs is not completely understood at present (17).

Numerous drugs, including ethanol and anesthetics are known to interact with the $GABA_A$-R (16, 18). Those drugs that potentiate the effects of GABA are sedative / hypnotic agents whereas those that inhibit the effects of GABA are convulsants.

3.1.1. Gene knockout of the beta 3 subunit of the $GABA_A$-R

The beta 3 subunit of the $GABA_A$-R is a key component of $GABA_A$-R isoforms in many regions of the brain, particularly cerebral cortex, hippocampus, hypothalamus, cranial nerve ganglia, and spinal cord (19-21). Production of beta 3 is even greater and more widespread in the prenatal and neonatal brain (20, 21).

Recently, mice lacking the beta 3 subunit of the $GABA_A$-R have been produced and characterized (22). Approximately 90% of mice homozygous for the mutation die as neonates and ~60% have cleft palate. GABA and benzodiazepine binding sites are both reduced ~50% in brains of homozygotes and $GABA_A$-R function is severely impaired (22, 23). Those homozygous mice that survive are runted until weaning, are hyperactive and hyperresponsive to human contact, have difficulty swimming, walking on grids, and frequently fall of platforms and rotarods (22). Thus, these mice are revealing fascinating roles for the beta 3 subunit in $GABA_A$-R function, palate formation, epileptogenesis, and behavior.

Recently, these mice have been examined to determine the contribution that beta 3 subunit containing $GABA_A$-Rs make to the response to various sedative / hypnotic drugs (24). In sleep time assays, these mice are less sensitive to etomidate and midazolam, but they are normally sensitive to pentobarbital and ethanol. In a tail clamp/withdrawal assay, these mice are less sensitive to the immobilizing effect of the volatile anesthetics halothane and enflurane, whereas in a loss of righting reflex assay sensitivity to the obtunding effect of these same drugs in unchanged. Such data support the hypotheses that: (a) separate components of the anesthetic state are mediated via different central nervous system loci (25); and, (b) different anesthetics produce anesthesia by different specific molecular interactions.

3.1.2. Gene knockout of the gamma 2 subunit of the $GABA_A$-R

Although alpha and beta subunits are able to coassemble to form functional GABA gated channels, the presence of a gamma subunit is required to confer sensitivity to benzodiazepines (26). The gamma 2 subunit is the major gamma subunit produced in the developing and adult brain and spinal cord (20, 21). Since most native receptors are thought to be pentamers that consist of two alpha subunits,

two beta subunits, and a gamma subunit (17, 27), gamma 2 is a constituent of the majority of $GABA_A$-Rs in the nervous system.

Gene targeting has been used to create mice that completely lack the gamma 2 subunit of the $GABA_A$-R (28). Most gamma 2 knockout mice die in the early neonatal period although a few survive for as long as 18 days. These mice appear relatively normal at birth, but sensorimotor abnormalities such as hyperactivity, impaired grasping and righting reflex, and abnormal gait soon develop. Radioligand binding revealed a nearly complete absence of benzodiazepine sites (~94% reduction) but only a minor reduction (~22%) in GABA sites. Electrophysiologic analysis demonstrated that the $GABA_A$-Rs that are present are composed primarily of alpha and beta subunits. These receptors respond normally to bicuculline, picrotoxin, and pentobarbital, but are completely unresponsive to the benzodiazepine flunitrazepam. A limited number of mice that survived the neonatal period were tested at postnatal day 14 for behavioral response to orally administered diazepam. A dose of this benzodiazepine that induced sedation and loss of the righting reflex in wild type mice, was without behavioral effects in the knockout animals. These results demonstrate the critical importance of the gamma 2 subunit to benzodiazepine sensitivity at the pharmacologic, electrophysiologic, and behavioral levels.

3.1.3. Gene knockout of the long splice variant of the gamma 2 subunit of the $GABA_A$-R

The gamma 2 subunit is known to exist in two variant forms produced by alternate splicing of the mRNA (29, 30). The long variant (gamma 2 long) differs from the short variant (gamma 2 short) by the presence or absence, respectively of a 24 base pair sequence derived from exon 9 of the gamma 2 gene. Some *in vitro* studies have clearly demonstrated that gamma 2 long is absolutely required for ethanol potentiation of GABA (31, 32). However, others have failed to observe this absolute requirement (33-35) and still others have observed the requirement is some expression systems but not in others (36). Thus, the importance of the splice variants to ethanol action at the $GABA_A$-R is highly controversial.

To investigate the physiologic and pharmacologic functions of the gamma 2 long splice variant, mice have been created that specifically and completely lack that subunit variant (37). To create these gene targeted animals, the 24bp exon which distinguishes the long from the short variant was deleted from the genome. In the absence of gamma 2 long, gamma 2 short is produced in increased quantities. These mice are viable, healthy, and overtly indistinguishable from wild type animals. A modest difference in anxiety was noted with knockouts displaying signs of increased anxiety compared to wild types. Of direct interest, knockout mice were ~20% more sensitive to the benzodiazepine midazolam and ~18% more sensitive to the benzodiazepine site agonist zolpidem. In contrast, sensitivity to other drugs that interact with the $GABA_A$-R such as pentobarbital and etomidate was unaffected. Because of the controversy surrounding the importance of gamma 2 long and ethanol action, these mice were extensively analyzed for changes in response to ethanol. Electrophysiologically, ethanol potentiated the effects of GABA to a similar extent in the presence (wild type) or absence (knockout) of gamma 2 long. Thus, in a neuronal context, native $GABA_A$-R do not have an absolute requirement for the long variant to bestow ethanol potentiation of GABA. Similarly, at the whole animal level, no differences were detected in ethanol induced sleep time, anxiolysis, hyperlocomotor activity, tolerance, or dependence.

3.1.4. Gene knockout of the alpha 6 subunit of the $GABA_A$-R

The alpha 6 subunit of the $GABA_A$-R is expressed almost exclusively in cerebellar granule cells of the adult brain; low levels of expression have been observed in dorsal cochlear nuclei and possibly inferior colliculi, substantia nigra and thalamus (38, 39). The alpha 6 subunit is atypical of $GABA_A$-R subunits in that it confers diazepam insensitive binding of Ro15-4513 to $GABA_A$-R isoforms. A point mutation in the alpha 6 subunit of the Alcohol Non-Tolerant rat line segregates with enhanced behavioral sensitivity to ethanol and other drugs that can modulate GABAergic activity (13, 40). The unique pharmacology of the alpha 6 subunit and the mutant rat line suggest a critical role for the alpha 6 subunit in whole animal behavioral response to sedative/hypnotic agents.

In an effort to better define the physiologic and pharmacologic functions of the alpha 6 subunit, two different groups of investigators created alpha 6 knockout mice (38, 41). Targeted disruption of the alpha 6 gene eliminated production of alpha 6 mRNA, protein, and diazepam insensitive binding of Ro15-4513 in the cerebellar granule cell layer of the mice. Unexpectedly, elimination of alpha 6 induced selective degradation of the delta subunit in cerebellar granule cells (38). Behaviorally, these mice are viable, fertile, and appear overtly indistinguishable from wild type animals.

Surprisingly, these knockouts do not differ from wild type mice in the hypnotic response to midazolam, ethanol, Ro15-4513, or pentobarbital (GEH, unpublished observations, and 41). Likewise, the obtunding and ataxic effects of halothane and enflurane are also unchanged (41). More recently, it has been demonstrated that alpha 6 homozygous mice also respond normally to ethanol in terms of acute functional tolerance, protracted tolerance, and withdrawal hyperexcitability (42). Thus, alpha 6 gene knockout mice have yet to reveal a significant phenotypic alteration of behavioral response to anesthetics and ethanol. This subunit therefore, appears to be of minor physiological significance to mice or its absence is compensated for by alterations in other subunits. Changes in cerebellar granule cell pharmacology suggest that the latter may have indeed occurred (43).

3.2. Serotonin receptors

Abnormal function of the serotonin (5-hydroxytryptamine, 5-HT) neurotransmitter system has been associated with behavioral abnormalities including suicidal tendencies, violence, anxiety, depression, aggression, and alcoholism. The main function of serotonin in the central nervous system is that of neuromodulation. Serotonin functions to alter the rate of firing of neurons or to modify the

release of other neurotransmitters such as GABA or dopamine. Serotonin exerts its effects by binding to membrane spanning receptors, of which 14 have been identified to date (44). With the exception of the 5-HT_3 receptors which are ligand gated ion channels, most 5-HT receptors are coupled to GTP-binding proteins and thus exert their effects by activation of second messenger systems.

3.2.1. Gene knockout of the serotonin 5-HT_{1B} receptor

The 5-HT_{1B} receptor is expressed in basal ganglia, central gray, hippocampus, and raphe nuclei (44). It has been posited that several drugs which inhibit aggressive behavior exert their effects through 5-HT_{1B} receptors. To test this, mice were produced in which the 5-HT_{1B} receptor was inactivated by gene targeting (45). These mice did not respond to the hyperlocomotor effects of the 5-HT_1 agonist RU24969. A most striking result of the genetic manipulation was that homozygous mutants displayed enhanced aggressive behaviors compared to controls. More recently, this mutation has been reported to alter startle reactivity, habituation, and prepulse inhibition (46).

Crabbe *et al.* (47) tested these 5-HT_{1B} receptor knockouts for several behavioral effects of ethanol. Targeted disruption of this gene modified the behavior of the mutant mice such that they voluntarily consumed twice as much ethanol as wild type mice. In addition, these knockouts were less sensitive to the ataxic effects of ethanol, and they did not develop tolerance to ethanol as quickly as wild type mice. The mutant mice did not differ in their preference for non-alcohol tastants, or severity of withdrawal following acute or chronic exposure to ethanol. These results demonstrate that the 5-HT_{1B} receptor is involved in regulating drinking behavior and sensitivity to some of the effects of ethanol. This study also highlights the notion that ethanol-related behaviors (such as consumption and withdrawal) may be controlled by independent mechanisms.

3.3. Opiate receptors

Opiates are an integral component of many anesthetic techniques, and are commonly used to produce the anesthetic state by themselves. The initial classification of opiate receptors was functional and based on responses to different opioid drugs (mu - morphine, kappa - ketocyclazocine, rho - N-allylnormetazocine, delta - enkephalins, epsilon - beta endorphins) (48-50). The subsequent application of molecular biology techniques has resulted in the cloning of mu, kappa, and delta opiate receptors (51-53), as well as a previously unrecognized receptor called the X-opioid receptor or the orphan opioid receptor (54, 55). All known opiate receptors mediate their effects via closely-linked G proteins, and probably use a variety of second messenger systems such as adenylate cyclase, potassium and calcium channels, and phospholipase C. Although the relative contributions of each of these receptor subtypes to different pharmacologic effects of opiates is still a matter of debate, the mu receptor has been implicated as a mediator of morphine-induced analgesia. The mu receptor has been classified into two subtypes, mu 1 and mu 2, based on relatively high and low binding affinity for opiate agonists such as naloxonazine and morphine, respectively (56). These two subtypes are believed to be mediators of different opioid effects (e.g., supraspinal analgesia versus respiratory depression), but the assignment of cloned receptors to either subtype remains controversial.

3.3.1. Gene knockout of the opiate mu receptor

Mice lacking mu receptors were created by disrupting the mu gene using homologous recombination techniques (57, 58). Null allele mice were viable, and morphologically and histologically identical to wild-type mice. In addition, their baseline locomotor activity, anxiety, learning ability, coordination, and habituation behaviors were similar to wild-type mice. However, binding of ^{3}H-DAMGO, a mu subtype specific opiate ligand, was essentially absent in null allele mice, and present at only 40% or 58% of baseline levels in heterozygous mice. Absence of mu receptor was further confirmed by immunostaining techniques. There were no compensatory changes in receptor number or binding affinity of kappa or delta subtypes as assessed by ^{3}H-U69,593/3H-CI-977 and ^{3}H-[D-Pen2,D-Pen5]enkephalin/^{3}H-naltrindole binding, respectively. The nociceptive responses of mice deficient in the mu subtype were altered both in the absence and presence of morphine. Hot plate tail flick latency (largely a measure of spinally-mediated analgesia) was decreased in heterozygous and null allele mice by approximately 25%, while the time to first paw lick (reflecting supraspinal analgesic mechanisms) was decreased by a similar magnitude. These results suggest that mu receptors play a role in the action of endogenous opiate-mediated nociception at both the spinal and supraspinal levels. The morphine dose-response relationship using these same nociceptive assays was shifted downward and to the left in heterozygous mice, and null allele mice had no response to morphine doses as high as 56 mg/kg, supporting a major role for mu receptors in morphine-induced analgesia. Interestingly, mu opiate receptors also appear to be required for delta agonists to be efficacious, since analgesia due to [D-Pen2,D-Pen5]enkephalin and the enkephalin hexapeptide Tyr-D-Ser(O-T-Bu)-Gly-Phe-Leu-Thr(O-T-Bu) (BUBU) was dramatically reduced in mice lacking mu receptors (57, 59). Other behaviors attributable to opiates such as rewarding behavior (measured with the place-conditioning paradigm) and dependence/withdrawal were also absent in mu deficient mice.

3.4. Alpha adrenergic receptor

Within the central nervous system, norepinephrine has substantial antinociceptive, sedative, and sympatholytic properties, and agonists of the alpha-2 receptor such as dexmetomidine or clonidine are used in clinical anesthesia practice for these purposes (60). The alpha-2 receptor exists as three different subtypes, alpha-2a, alpha-2b and alpha-2c. Similar to the opiate receptor, the alpha-2 receptor is coupled to a G protein system for further signal transduction. However, in contrast to the opiate receptor, the lack of sufficiently selective subtype-specific ligands has impeded efforts to dissect the relative importance of alpha-2 subtypes to various pharmacologic responses. Gene targeting has provided substantial insight to alpha-2 adrenergic receptor subtype pharmacology via animals with point mutations of the alpha-2a adrenergic receptor, and those lacking alpha-2b and alpha-2c adrenergic receptors, and overexpressing alpha-2c adrenergic receptors.

3.4.1. Point mutation of the alpha-2a adrenergic receptor

Production of mice (called D79N mice) which are homozygous for a point mutation of the aspartate residue to asparagine at position 79 of the alpha-2a adrenergic receptor has enabled elucidation of the role of this receptor subtype in homeostatic and anesthetic processes (61). This mutation eliminates allosteric modulation of the alpha-2 receptor by monovalent cations (62) and perturbs G protein coupling (for example, coupling to activate potassium channels) (63). Despite normal levels of mRNA encoding the alpha-2a receptor, the density of alpha-2a receptor is decreased in mutant mice (approximately 80% of wild type density); however, affinity for agonists is increased due to the loss of allosteric modulation previously mentioned.

Pharmacologic responses to alpha-2 receptor agonists were markedly different in wild type and mutant mice. Although baseline hemodynamics were similar in wild type and D79N mice, the hypotensive response to alpha-2 receptor agonists such as dexmetomidine was absent in the latter. Dexmetomidine reduced coordination (as measured by rotarod performance) and induced sleep in wild type but not mutant mice (64). Similarly, dexmetomidine decreased halothane requirement (as measured by loss-of-righting reflex) and increased the pain threshold (as measured by the ramped hot plate test, a measure of supraspinal pain perception) in wild type but not mutant mice. In contrast, non-alpha-2 receptor agents such as pentobarbital, the adenosine A1 receptor agonist R-(2-phenylisopropyl)adenosine, and morphine produced equivalent hypnotic, anesthetic-sparing, and analgesic responses, respectively, in wild type and mutant mice. These data confirm that the alpha-2a receptor is the primary mediator of the hypnotic, anesthetic-sparing, and analgesic properties of alpha-2 receptor agonists.

Alpha-2 receptor agonists exert analgesic effects when administered at the spinal level, and also synergistically enhance the spinal analgesia mediated by spinally-administered opioids. The spinal anti-nociceptive properties of alpha-2 receptor agonists in D79N mice have also been investigated (65). Intrathecal administration of UK 14,304 (a nonsubtype selective alpha-2 receptor agonist) fails to induce analgesia as measured by the tail flick test in D79N mice, while the analgesic properties of intrathecally-administered morphine were preserved, confirming that the alpha-2a receptor subtype is the primary mediator of alpha-2 adrenergic spinal analgesia. Alpha-2 receptor agonists inhibited the classic nociceptive behavior following the intrathecal administration of substance P in both wild type and D79N mice, but the dose required in D79N mice was several orders of magnitude greater than that required in wild type mice. Synergy of alpha-2 adrenergic and opioid effects require activation of delta opioid receptors, and were investigated after co-administration of the delta opioid agonist deltorphin II, and a cocktail of UK 14,304 and prazosin (which blocks alpha-1, alpha 2b, and alpha-2c adrenergic receptors, resulting in relatively pure alpha-2a agonism). Deltorphin II potency was similar in wild type and D79N mice, whereas the alpha-2 cocktail was 2 orders of magnitude less potent in D79N mice than in wild type mice. Isobolographic analysis of responses after co-administration of deltorphin and equipotent doses of alpha-2 cocktail revealed marked synergy of alpha-2 adrenergic and opioid effects in wild type but not in D79N mice. These data confirm that the alpha-2a adrenergic receptor is required for synergy of alpha-2 adrenergic agents and opioids. Finally, the ED_{50} of morphine required to inhibit the response to substance P was 75 times greater in D79N than in wild type mice, and was not affected by co-administration of the alpha-2a antagonist idaxozan as was the case in wild type mice. This suggests that the alpha-2a subunit is crucial to the modulation of morphine-induced antinociception by descending spinal adrenergic pathways.

3.4.2. Gene knockout of the alpha-2b adrenergic receptor

Mice lacking the alpha-2b adrenergic receptor have been produced by homologous recombination (66). The alpha-2b adrenergic receptor apparently mediates the initial hypertensive response to alpha-2 adrenergic agonists, and ameliorates the subsequent hypotension, because the former is not observed in mice lacking the alpha-2b adrenergic receptor, and the hypotension after alpha-2 adrenergic agonists is greater than in wild type animals. Unfortunately, no specific tests of anesthetic sensitivity have been published in alpha-2b null allele mice, although meeting summaries indicate that the anesthetic-sparing properties of dexmetomidine are unaltered in alpha-2b null allele mice, suggesting that the alpha-2b subtype is not integral to alpha-2 agonist anesthesia responses (67).

3.4.3 . Gene knockout and overexpression of the alpha-2c adrenergic receptor

Mice lacking the alpha-2c adrenergic receptor have also been produced by homologous recombination (66). Null allele mice are viable, fertile, and grossly normal, despite total absence of the alpha-2c-adrenoreceptor and marked reductions in ^{3}H-rauwolscine binding in brain areas where the alpha-2c-adrenoreceptor is found. Expression of alpha-2a and alpha-2b receptors is unaltered. Locomotor activity and hemodynamics after dexmetomidine administration are not different in null allele mice (68). The hypothermic effect of dexmetomidine is attenuated in null allele mice, while it is accentuated in mice which overexpress the alpha-2c receptor three-fold (69). Similar to the case for alpha-2b null allele mice, preliminary reports indicate that alpha-2c null allele mice retain an anesthetic-sparing response to dexmetomidine, suggesting that the alpha-2c subtype is not integral to alpha-2 agonist anesthesia responses (67).

3.5. Dopamine receptors

Dopamine is the chief catecholamine in the mammalian central nervous system, and is integral to regulation of locomotor activity, motion, affect, and neuroendocrine function. Insufficient dopaminergic activity due to neuronal degeneration contributes to Parkinson's disease, and dopamine receptor antagonists are important agents in the control of schizophrenia. The initial functional classification of dopamine receptors into D1 (stimulating adenylate cyclase activity) and D2 (inhibiting adenylate cyclase) has been superseded by the subsequent discovery and cloning of five dopamine receptor subtypes (70). The D1 and D5 subtypes share a significant sequence homology, and correspond to the old "functional D1" classification. The D2,

D3, and D4 subtypes also share sequence homology and correspond to the "functional D2" classification. All of these subtypes are coupled to G protein effector systems. D1 and D5 subtypes stimulate and D2, D3, and D4 inhibit adenylate cyclase as their major signaling pathway, but these receptor subtypes are probably also capable of using other second messenger systems.

Although central dopaminergic receptors do not appear to be integral to the production of anesthesia, they have been implicated in the behavioral response to addictive drugs such as opiates, especially the rewarding properties of opiates and the somatic expression of opiate abstinence.

3.5.1. Gene knockout of the dopamine D1 receptor

Mice lacking the dopamine D1 receptor have been produced by targeted gene disruption (71). Null allele mice are growth retarded and die after birth unless fed a special diet. Their locomotor behavior and coordination is normal, but rearing behavior is decreased. No specific data concerning anesthetic responses are available.

3.5.2. Gene knockout of the dopamine D2 receptor

Mice lacking the dopaminergic D2 receptor have been produced by disrupting the dopamine D2 receptor gene (72, 73). Null allele mice exhibited decreased spontaneous locomotor activity, but normal exploratory behavior and habituation (74, 75). Morphine and RB 101 (an inhibitor of enkephalin degrading enzymes) induced a similar hyperlocomotor response in mice with and without the D2 receptor. The rewarding effect of morphine (measured by the place-conditioning paradigm) was absent in mice lacking the D2 receptor, and this response was not due to increased endogenous enkephalin levels, since the same result was observed with a substantially greater morphine dose (9 mg/kg). Incentive behavior to food was preserved, indicating that the defect was specific. Activation of the D2 receptor decreases the severity of withdrawal symptoms, and D2 blockade induces withdrawal in morphine dependent animals. However, mice with and without the D2 receptor did not differ in their withdrawal responses, indicating that although they may be involved in the withdrawal process, D2 receptors are not required for withdrawal to be manifest. No specific data regarding anesthetic responses are available.

A recent study of the D2 receptor knockout mice (75) highlights an important aspect of knockout studies that has only recently received serious attention, namely the contribution of genetic background to the phenotype of knockout animals (for discussion, see:76). Analysis of the effects of genetic background on the D2 receptor knockout has revealed a significant performance deficit in one of the two inbred parental mouse strains (Strain 129) that was used to create the knockout animals. Inbred Strain 129 mice had very low levels of spontaneous activity, exploratory activity, and poor rotarod performance, just like the D2 receptor knockouts. The exact contribution of the Strain 129 genome versus the dopamine D2 receptor knockout to the phenotypes being studied was not conclusively established though Kelly *et al.* (75) address, at length, the issue of genetic background. It remains to be determined if the changes in morphine responsiveness mentioned above (74) are similarly confounded by effects of the genetic background.

3.5.3. Gene knockout of the dopamine D3 receptor

Mice lacking the dopaminergic D3 receptor have been produced by introducing a premature chain-termination mutation in the D3 gene (77). Binding of ^{125}I-iodosulpride to D3 receptors was absent in null allele mice. Mice lacking the D3 receptor exhibited increased locomotor activity and rearing behavior. Unfortunately, no data on specific sedative/anesthetic responses appears to be available.

3.5.4. Gene knockout of the dopamine D4 receptor

The dopamine D4 receptor has recently been intensively investigated. Interest in the D4 receptor has been stimulated by the observations that D4 receptors displays very high affinity for the antipsychotic clozapine (78), D4 receptors may be increased in abundance in brains of schizophrenics (79), D4 receptors are highly polymorphic (80), and particular polymorphisms have been associated in humans with alcoholism (81-83), novelty seeking behavior (84, 85), and opiate abuse (86), and in mice with ethanol drinking (87).

To assess the functional significance of the D4 receptor *in vivo*, mutant mice were produced in which the D4 receptor gene was inactivated by gene targeting (88). The mutant mice were overtly indistinguishable from controls, however, they were less sensitive to clozapine. Dopamine synthesis and turnover was increased in brains of the knockouts. On tests of exploratory behavior in both novel and familiar environments, D4 receptor mutant mice exhibited reduced locomotor activity. Surprisingly, on a test of complex motor coordination, the rotarod, the mutant mice performed significantly better than their wild type littermates. When tested for the hyperlocomotor effects of ethanol, cocaine, and methamphetamine, it was observed that the D4 receptor knockouts were supersensitive to these drugs. Although the molecular and cellular mechanisms by which the D4 receptors modulate the behaviors that are altered in the knockout animals have yet to be definitively established, it is clear that the D4 receptors are important for normal and drug stimulated motor behaviors.

4. KINASES

Phosphorylation / dephosphorylation is one of the primary mechanisms of ion channel modulation. Cellular studies have shown that the functions of both N-methyl-D-aspartate receptors (NMDA-Rs, a glutamate-gated Ca2+ channel) and $GABA_A$-Rs are modulated by phosphorylation (89). Two kinases that phosphorylate NMDA-Rs and $GABA_A$-Rs, fyn kinase and the gamma isoform of protein kinase C (PKC), respectively, were deleted in mice in separate experiments initially to determine the role these kinases have in long-term potentiation and learning and memory (90, 91). Subsequently, their roles in mediating the intoxicating effects of ethanol were investigated.

4.1. Gene knockout of fyn tyrosine kinase

Fyn tyrosine kinase, a non-receptor tyrosine kinase, was inactivated in mice by gene targeting (90). Mutant mice were reported to have defective long-term potentiation. Testing in the Morris water maze revealed that the fyn mutant mice were incapable of recalling previous training sessions and using that information to escape from the pool. Fyn null mice also failed the water maze transfer test (no quadrant

preference). Histologically, a clear defect in the arrangement of the granule cells of the dentate gyrus and the pyramidal cells of the CA3 region was observed.

Miyakawa *et al.* (92) investigated the role of fyn kinase in modulating the behavioral effects of ethanol. Fyn knockout mice slept twice as long as control mice after ethanol administration. Mechanistically, it appears that in the absence of fyn kinase, ethanol stimulated phosphorylation of NMDA receptors is ablated. This results in a failure of hippocampal NMDA receptors to develop acute tolerance to ethanol, ultimately modulating whole animal response to the hypnotic effects of ethanol.

4.2. Gene knockout of the gamma isoform of protein kinase C

PKC-gamma is one of 11 isoforms of PKC (93), and is expressed only post-natally (94). As with the fyn knockout, PKC-gamma was deleted in order to determine the role of this central nervous system-specific isoform of PKC in learning and memory (91). PKC-gamma null animals were viable and displayed normal behaviors for mating, grooming, feeding, and circadian activity. However, PKC-gamma knockout animals had an abnormal gait and were more prone to falling off an inclined pole than wild-type controls. Long-term potentiation and spatial learning were abnormal in the mutant mice.

Ethanol induced sleep time was reduced in the PKC-gamma knockout animals by nearly 40% (95). In addition, ethanol induced hypothermia was reduced in knockouts compared to wild type animals. Because the $GABA_A$-R is a potential target of PKC-gamma mediated phosphorylation, and phosphorylation of the gamma-2 long splice variant of the $GABA_A$-R may be important for ethanol potentiation of GABA action (32), $GABA_A$-R function was assessed with ^{36}Cl flux assays using isolated brain membrane vesicles derived from cerebellum or cortex of the mice. These studies revealed no differences between genotypes in basal or muscimol-stimulated ^{36}Cl- flux. However, 15mM ethanol enhanced muscimol stimulated ^{36}Cl- flux by 30% in control animals, whereas no enhancement by ethanol was observed in knockout cerebellar vesicles and a significant reduction was seen in knockout cortical vesicles. Although these studies established a clear role for PKC-gamma in mediating some of the behavioral effects of ethanol, it remains to be determined which specific cellular proteins (e.g., $GABA_A$-Rs and others) the kinase actually interacts with to elicit these behavioral changes in ethanol sensitivity.

5. NEUROTRANSMITTERS

5.1. Nitric Oxide

Nitric oxide is a labile gas that functions as a cellular messenger. In the nervous system, nitric oxide functions as a neurotransmitter. In contrast to other neurotransmitters, there is no known mechanism for storage of nitric oxide; it is synthesized *de novo* as needed from L-arginine by nitric oxide synthase (NOS). Three NOS genes and the enzymes they encode have been identified. Neuronal and endothelial NOS are constitutively produced whereas macrophage NOS is inducible. Knockout mice have been created that harbor inactivated neuronal (96), endothelial (97), and macrophage (98, 99) NOS genes. Only neuronal NOS knockouts will be covered here as this is the only isoform of NOS that is likely to be involved in mediating the effects of alcohol and anesthetics.

5.1.1. Gene knockout of neuronal nitric oxide synthase

Neuronal NOS is expressed in many neurons of the central and peripheral nervous systems. Mice harboring a targeted disruption of the neuronal NOS gene are viable, fertile, and lack histopathological changes in the central nervous system (96). The mutant mice have grossly enlarged stomachs which is accompanied by hypertrophy of the circular muscle layer of the stomach and pylorus. Presumably these defects are due to the lack of nitric oxide in the neurons of the peripheral nervous system which innervate this part of the digestive tract. Mice lacking neuronal NOS also display enhanced aggression and inappropriate sexual behaviors (100).

Involvement of nitric oxide in anesthesia has been suggested from studies employing inhibitors of NOS. It has been reported that pharmacologic inhibition of NOS results in reduction in the minimum alveolar concentration for halothane anesthesia in rats (101), although others have not observed this effect (102). To directly test the role of neuronal NOS, isoflurane minimum alveolar concentrations and loss of righting reflex EC_{50}'s were compared between wild type and neuronal NOS knockout mice (103). Surprisingly, isoflurane requirement was unaltered by the neuronal NOS knockout. However, the same authors also reported that pharmacologic inhibition of NOS in wild type, but not knockout mice, reduced isoflurane requirement. These results suggest that the reduction in anesthetic requirement by NOS inhibitors is indeed mediated by neuronal NOS. Furthermore, the lack of a change in anesthetic requirement in the absence of a NOS inhibitor in the knockout mice implies that changes occurred in the nervous system that compensate for the lack of neuronal NOS. A similar conclusion was reached in a study of nociception-induced neuroplasticity in the neuronal NOS knockout mice. While wild type and knockouts did not differ in formalin-induced nociceptive behavior, administration of a NOS inhibitor blocked the response in wild type mice but was ineffective in the knockouts (104). Unknown compensatory changes such as these are a limitation of conventional knockout technology.

6. PERSPECTIVES

The application of genetically engineered animals to dissect the mechanisms of action of alcohol and anesthetics is only just beginning. The recent results highlighted above demonstrate the vast power and utility of this approach. Already, genetically altered animals have led to exciting advances in our understanding of how these drugs exert their effects *in vivo* at the whole animal level. It is clear that many neuronal signaling pathways are involved. Transgenic technologies coupled with more traditional types of analysis (e.g., molecular, electrophysiologic, etc.), are expected to lead to rapid progress in unraveling the complexities of alcohol and anesthetic action.

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