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The Role of Connexin-36 Gap Junctions in Alcohol Intoxication and Reward

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THE ROLE OF CONNEXIN-36 GAP JUNCTIONS IN ALCOHOL INTOXICATION AND REWARD

by

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found satisfactory.

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BRIGHAM YOUNG UNIVERSITY

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ABSTRACT

THE ROLE OF CONNEXIN-36 GAP JUNCTIONS IN ALCOHOL INTOXICATION AND REWARD

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The purpose of this thesis project was to examine the function of connexin-36 (Cx36) gap junctions (GJs) in producing alcohol's intoxicating and rewarding effects. GABA neurons are thought to inhibit dopamine (DA) neurotransmission in the mesocorticolimbic system, which originates in the midbrain ventral tegmental area (VTA) and projects to limbic structures such as the nucleus accumbens (NAcc). The mesolimbic DA system is believed to be the neural substrate of alcohol intoxication and addiction (Tepper, Paladini, & Celada, 1998). Alcohol suppresses the firing rate of GABA neurons in the VTA (Gallegos, Criado, Lee, Henriksen, & Steffensen, 1999) and presumably disinhibits DA neurons thereby resulting in enhanced release of DA in the NAcc. Interestingly, VTA GABA neurons appear to form part of a larger syncytium of GABA neurons in the reticular formation that are linked by electrical synapses via Cx36

GJs (Allison, et al., 2006; Stobbs, et al., 2004; Lassen, et al., 2007). Gap junction blockers, including the Cx36-selective antagonist mefloquine, also suppress the excitability and electrical coupling of VTA GABA neurons (Stobbs, et al., 2004). Thus, I hypothesized that Cx36 GJs cause synchrony in VTA GABA neurons which alcohol blocks to cause intoxication and reward.

To accomplish these studies I compared the effects of intoxicating doses of ethanol in Cx36 knockout (KO) mice and mefloquine-treated mice and their wild-type (WT) controls with two tests that index ataxia, an open field activity system and the fixed-speed rotarod apparatus, as well as with ethanol self-administration. I found that Cx36 KO and mefloquine-treated mice exhibit significantly more ethanol-induced loss of movement in the open field test, a paradigm which indexes gross motor activity and tremor, but less ataxia than their WT controls in the rotarod paradigm, a paradigm which indexes balance and coordination. Most importantly, both Cx36 KO and mefloquine-treated mice consumed less ethanol than their controls. These findings provide evidence in support of the hypothesis that Cx36 GJs are important targets for ethanol effects in the mesolimbic system.

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TABLE OF CONTENTS

LIST OF FIGURES

THE ROLE OF CONNEXIN-36 GAP JUNCTIONS IN ALCOHOL INTOXICATION AND REWARD

Kathryn D. Bradley

INTRODUCTION

In the 2007 National Survey on Drug Use and Health, 7.2 million people ages 12-20 reported themselves as binge drinkers and 2.3 million as heavy drinkers (Kennet, 2008). Of those ages 18-26, 41.8% were binge drinkers and 14.7% were heavy drinkers. Over half of Americans surveyed, or 126.8 million people, report currently using alcohol. In 2000, it was estimated that drug and alcohol abuse cost the United States economy $184.6 billion dollars (Harwood, 2000). $226 million of that amount is directed toward alcohol abuse research. While there are some benefits from controlled alcohol use, excessive and addictive drinking can lead to a diversity of social and physical problems. Excessive alcohol drinking can cause nutritional deficits (Rehm, Room, & Graham, 2003), hypertension, cirrhosis, and disruption of proper neuronal development, among many other problems (Wilson, 2009). Alcoholism is significantly associated with colon and rectal cancer (Dimitrijevic, Kalezic, Ristic, Bojovic, & Dimitrijevic, 2008). Alcohol hinders the function of neural stem cells and may alter gene expression (Ishii, et al., 2008). In other words, alcohol is ever-present in American society that needs to be fully understood.

While many of alcohol's mechanisms of action have been revealed through research, there is still much to discover. Research shows a mixed influence of genetics

and the environment in alcohol addiction, including a genetic role in neuronal disinhibition (Schuckit, 2009). Treatment for alcohol addiction ranges from therapies that treat environmental causes such as musical interventions (Mays, Clark, & Gordon, 2008), meditation (Galanter, 2006), and organized programs like Alcoholics Anonymous (Borkman, 2008) to pharmaceutical therapy treating the biological causes, using drugs like naltrexone and acamprosate, that aim to block reward mechanisms (Garbutt, 2009). Most likely, the best treatment would be a combination of both methods to approaching alcoholism. Only by discovering all of alcohol's mechanisms of action can anyone hope to develop an effective treatment for alcohol addiction (Mukherjee, Das, Vaidyanathan, & Vasudevan, 2008).

Understanding the biology of alcohol intake may also expose biomarkers that predict a person's predisposition to alcohol addiction. Such an approach could help avoid some unnecessary suffering. Some predictors of a potential alcohol addiction have already come to light. Childhood ADHD (Knop, et al., 2009) and use of cigarettes (Merline, Jager, & Schulenberg, 2008), are two candidates for addiction prediction. However, specific biomarkers would ideally be able to show genetic predisposition (Peterson, 2004-2005). Those can only be discovered by understanding all the neurological effects of alcohol. Alcohol research is already a well studied field yet there is still much to discover. I have examined the role of Cx36 GJs in alcohol intoxication and reward. I have done this by testing wild type mice and mice whose Cx36 gene have been knocked-out on various behavioral tests while under the influence of alcohol.

BACKGROUND

Mesocorticolimbic Dopamine System and Drug Abuse

The commonly accepted theory behind the addictive properties of alcohol holds that an increase in DA release in the mesocorticolimbic system is responsible for the rewarding effects of alcohol (Wise, 2004). The mesocorticolimbic system consists of the circuitry connecting the hippocampus, amygdala, medial prefrontal cortex, nucleus accumbens (NAcc) and Ventral Tegmental Area (VTA), though the latter two are considered the main reward centers in this system (Pierce & Kumaresan, 2006). The VTA consists of two kinds of neurons projecting to the NAcc: Excitatory DAergic neurons and inhibitory GABAergic neurons. Supporting the aforementioned DA theory, rats will self-administer ethanol (EtOH) directly into the VTA (Gatto, McBride, Murphy, Lumeng, & Li, 1994) and microdialysis reveals an increase in DA levels (Di Chiara & Imperato, 1985). However, rats continue to self-administer alcohol and opioids after neurotoxic-lesioning of the DA neurons (Koob, 1992), limiting the role of the DA system alone to psychostimulant reward.

GABA Neurons in the Mesocorticolimbic System

It has been shown that GABAergic neurons hold an inhibitory control over DA neurons in the midbrain substantia nigra (Tepper, et al., 1998). GABA neurons in the midbrain VTA would likely have the same effect, as they are analogous DA systems. However, we have previously demonstrated that GABA neurons in the VTA are not just local circuit interneurons, as they project to cortical structures (Steffensen, et al., 1998). Most importantly, EtOH suppresses the firing rate of VTA GABA neurons (Gallegos, et

al., 1999). Indeed, they are an order of magnitude more sensitive to EtOH than DA neurons. In that light, if EtOH were to block GABA neurons, preventing them from its spontaneous firing activity, alcohol would in effect disinhibit DA neurons, causing an increase in DA release and ultimately, reward. This physiological pathway fits in with the evidence of the DA model for alcohol intoxication and reward, but reveals that GABA neurons are the main contributor, and arguably the neuronal transducer, of the intoxicating and rewarding properties of alcohol.

Electrical synapses

Research in the Steffensen lab is devoted to the characterization of the role of GABA neurons in the ventral tegmental area (VTA) in EtOH intoxication, reward, dependence and addiction. Previous studies from this lab have shown evidence for electrical coupling between VTA GABA neurons via GJs, and in particular, Cx36 GJs. For example, stimulation of the internal capsule (IC) in rats has been shown to elicit post-stimulus VTA GABA neuron spike discharges (ICPSDs) (**Fig. 1A**)(Steffensen, et al., 1998). Notice, in this exemplary intracellular recording from a single GABAergic interneuron, its multiply-spiking nature. Furthermore, acute intoxicating doses of EtOH (**Fig1B**), and non-specific GJ blockers, like quinidine, or the Cx36 GJ selective blocker mefloquine, suppress these ICPSDs (similar to **Fig1B**; (Stobbs, et al., 2004)), meaning that GABA neurons may be connected by Cx36 GJs. Although not shown here, *in situ* microelectrophoretic application of DA couples VTA GABA neuron spikes via a Cx36 GJ-mediated coupling. Further, VTA GABA neurons express Cx36 transcripts and dye couple suggesting that they are electrically coupled together in a network of sorts in the VTA and surrounding structures.

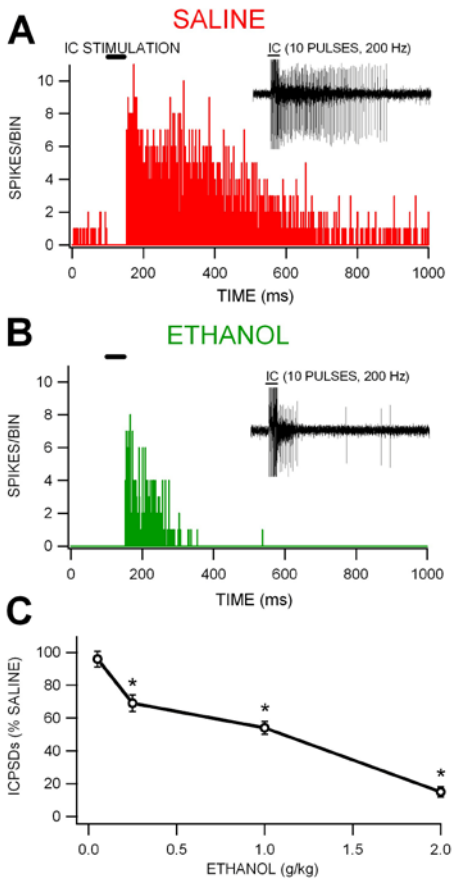

Figure 1. *Acute EtOH blocks internal capsule-induced VTA GABA neuron post-stimulus discharges (ICPSDs)* (A) The inset shows a representative recording of VTA GABA neuron spikes following brief, high frequency (10 pulses; 200 Hz) stimulation of the internal capsule in a halothane-anesthetized rat. VTA GABA neuron spikes not only accompany each internal capsule stimulation pulse, but are elicited for hundreds of msec after the stimulus train has ended. This particular set of stimulation parameters induced approximately 60 VTA GABA neuron ICPSDs, a level that produces approximately half-maximum PSDs. The peri-stimulus spike histogram (PSH) shows the effects of intraperitoneal saline control on VTA GABA neuron ICPSDs (average of 12 stimulation trials). (B) The representative trace and PSH (normalized to number of control stimulation trials) show the effects of intraperitoneal EtOH on VTA GABA neuron ICPSDs, and demonstrates that acute EtOH suppressed VTA GABA neuron ICPSDs. (C) This graph summarizes the effects of acute systemic EtOH on VTA GABA neuron PSDs. EtOH (0.25-2.0 g/kg) significantly reduced VTA GABA neuron ICPSDs compared to saline control, at doses that also significantly reduced firing rate (data not shown). Asterisk * indicates significance levels P<0.005. (Stobbs, et al., 2004).

GJs are pores formed by connexin subunits which physically couple two neurons, allowing current to pass through the junction in both directions, as well as permitting low-pass filtering (Willecke, et al., 2002). The evidence postulates that GJs couple GABA neurons allowing electrical current to reverberate through the network of neurons, which manifests as the multiply-spiking ICPSDs. As a further confirmation of a physical coupling between VTA GABA neurons, when GABA neurons are injected with Neurobiotin, a fluorescent dye, the dye is also found in not only neighboring but distant GABA neurons (Allison, et al., 2006).

These electrical synapses are sensitive to the voltage difference across the GJ membrane, depending on the connexin subunits that make up the GJ (Moreno, Rook, Fishman, & Spray, 1994). They also vary in their permeability to ions and small molecules (less than one kD) (M. V. Bennett, 2002; Qu & Dahl, 2002). The GJ's ability to transmit current and molecules provides a means of intercellular communication for both the developing and mature nervous system (M. V. Bennett, 2002; Roerig & Feller, 2000; Rozental, Giaume, & Spray, 2000). In particular, during development, GJs help to associate functional groups of cells before synapses are established (Leung, Unsicker, & Reuss, 2002; Nadarajah, Jones, Evans, & Parnavelas, 1997; Roerig & Feller, 2000; Rozental, et al., 2000).

Because GJs can transmit electrical information, they've been linked to the generation of high-frequency oscillations (LeBeau, Towers, Traub, Whittington, & Buhl, 2002; N. Maier, et al., 2002; Traub, et al., 2002). Further, GABA neuron networks regulate these oscillations through the network's GJs (Buzsaki & Chrobak, 1995; Galarreta & Hestrin, 2001; Tamas, Buhl, Lorincz, & Somogyi, 2000). Although most research on oscillatory patterns mostly focus upon the limbic system, hippocampus, thalamus, and neocortex, studies reveal the potential role of the midbrain in discharging rhythmic oscillations (Kitai, Shepard, Callaway, & Scroggs, 1999; Overton & Clark, 1997).

Through various second messenger systems, neurochemicals have been shown to modulate GJ electrical synapses, including GABA and monoamines (Qu and Dahl 2002). In regard to addiction, GJ exist in several structures of the reward pathway: the nucleus accumbens, striatum (Moore & Grace, 2002), hippocampus, and locus coeruleus

(Oyamada, Andrzejewski, Muckenhoff, Scheid, & Ballantyne, 1999; Srinivas, et al., 1999).

Many studies have linked GJs to the pathology of many CNS disorders. Some include epilepsy (Carlen, et al., 2000; Perez Velazquez & Carlen, 2000; Traub, et al., 2001), excitotoxicity (Ozog, Siushansian, & Naus, 2002), demyelination-induced neuropathies (Martini, Zielasek, & Toyka, 1998), ischemia-reperfusion (Frantseva, Kokarovtseva, & Perez Velazquez, 2002; Rami, Volkmann, & Winckler, 2001), and non-syndromic hearing loss (Kikuchi, Adams, Miyabe, So, & Kobayashi, 2000; Rabionet, Lopez-Bigas, Arbones, & Estivill, 2002). The diversity of the GJ's influence on so many diseases points to a crucial role of GJs in normal physiology (Nadarajah & Parnavelas, 1999; Simon & Goodenough, 1998; White, Deans, Kelsell, & Paul, 1998).

Connexins

Although attempted by many researchers, determining the structural and functional interactions of connexin proteins has proven illusive (Sosinsky & Nicholson, 2005). What is known of a connexin's topology is that the protein contains four hydrophobic regions, which translates to four transmembrane regions (Milks, Kumar, Houghten, Unwin, & Gilula, 1988). Two extracellular loops allow for docking with another hemichannel (six ring-forming connexins), while the N- and C-terminals and another loop lie intracellularly (Kumar & Gilula, 1996). In mammals, there are more than 20 family members of connexins (Willecke, et al., 2002).

Two types of connexins have been definitively shown to be expressed in neurons: Cx36, found in the neurons of the CNS (Belluardo, et al., 2000; D. F. Condorelli, N.

Belluardo, A. Trovato-Salinaro, & G. Mudo, 2000; M. R. Deans, J. R. Gibson, C. Sellitto, B. W. Connors, & D. L. Paul, 2001; Degen, et al., 2004; X. B. Liu & Jones, 2003), and Connexin45, expressed widely through the nervous system during development and less so as an adult (Maxeiner, et al., 2003; Zhang & Restrepo, 2002). Other connexins are suspected to reside in neurons but studies show mixed results (Micevych & Abelson, 1991; Nadarajah, Thomaidou, Evans, & Parnavelas, 1996; Solomon, Halat, El-Maghrabi, & O'Neal, 2001; Venance, et al., 2000); (Rash, Yasumura, Dudek, & Nagy, 2001). For example, Connexin32 has been repeatedly shown to be associated with oligodendrocytes (Dermietzel, et al., 1997; Li, Hertzberg, & Nagy, 1997; Rash, et al., 2001). As for a neuronal relationship, however, some studies associate connexin32-immunoreactivity to neuronal cell types (Dermietzel, et al., 1989; Nadarajah, et al., 1996; Nagy, et al., 1988; Oguro, et al., 2001; Priest, Thompson, & Keller, 2001; Reyher, et al., 1991; Yamamoto, Shiosaka, Whittaker, Hertzberg, & Nagy, 1989), while other studies question the reliability of the anti-connexin32 antibodies (Li, et al., 1997).

Connexin36 (Cx36)

To narrow in on the type of GJ responsible for the coupling of neurons causing ICPSDs in VTA GABA neurons, Mefloquine, a Cx36-selective blocker, was injected into rats while recording ICPSDs (Allison, et al., 2006). ICPSDs were blocked by this selective Cx36 antagonist, even up to 3 days post injection. Quinidine also blocks Cx36 in a reversible and concentration-dependent manner (Srinivas, Hopperstad, & Spray, 2001). However, it is not selective for Cx36 GJs or specific for GJs. Nonetheless, in one study, quinidine inhibited epileptiform transients in the hippocampus (Uusisaari, Smirnov, Voipio, & Kaila, 2002), supposedly due to the blockade of electrical synaptic

transmission between GABAergic inhibitory neurons (Galarreta & Hestrin, 2001; Tamas, et al., 2000; Yang & Michelson, 2001).

Cx36 is the main neuronal connexin found in the human central nervous system (Belluardo, et al., 2000). As determined by in situ hybridization, the areas with the highest concentration of Cx36 in humans are found in the inferior olivary complex, specific cerebellar cortex cells, the dentate gyrus, some hippocampal subfields, and the spinal cord gray matter (Belluardo, Trovato-Salinaro, Mudo, Hurd, & Condorelli, 1999). This is similar to the Cx36 pattern in the rat. There are also large populations of Cx36 found in the olfactory bulb, retina, anterior pituitary, and pineal gland (Belluardo, et al., 2000; Christie, et al., 2005). Because Cx36 is found so ubiquitously throughout the brain, it is expected that there are unknown electrical synapses throughout the central nervous system (Connors & Long, 2004). Generally, brain Cx36 levels peak two weeks after birth and fall steeply through the following week (Belluardo, et al., 2000). It is found in dispersed locations and may be involved in the broad neuronal coupling that networks the brain in early development. Cx36 chiefly couples GABAergic neurons (Deans, et al., 2001; Johansson, 2006). This happens in both juvenile and mature neocortex, connecting GABAergic dendrites to each other and connecting dendrites to somata (Hestrin & Galarreta, 2005). These connections allow for a synchronous network of electrical activity. Cx36 generates this synchrony of neurons via sharp wave-ripple (~200 Hz) formations (Nikolaus Maier, et al., 2002). Cx36 may contribute to these ripple patterns through gamma oscillations (Buhl, Harris, Hormuzdi, Monyer, & Buzsaki, 2003).

The Cx36 sequence is highly conserved: 98% identity with mouse and rat, 80% for the ortholog perch and skate (Belluardo, et al., 1999). The Cx36 gene's chromosomal location is on band 15q14 (Belluardo, et al., 1999). There are 71 base pairs after the initiation site in the Cx36 gene that includes one intron (Belluardo, et al., 1999; Condorelli, Belluardo, Trovato-Salinaro, & Mudo, 2000). Cx36 is a 321 amino acid chain with a 99 amino acid cytoplasmic loop (Condorelli, et al., 2000).

Research has shown several roles for Cx36. Cx36 GJs are involved in the rod pathway, specifically between amacrine and bipolar cells (Guldenagel, et al., 2001). Cx36 GJs in the suprachiasmatic nucleus help regulate circadian rhythms (Long, Jutras, Connors, & Burwell, 2005). Ablation of Cx36 results in memory deficits and coordination learning impairment (Frisch, et al., 2005). In rats that self-administer cocaine, modification of Cx36 mRNA levels occurs, varying on time and brain region, including a decrease in the prefrontal cortex. This may in part explain behavioral changes found in these rats (McCracken, et al., 2005). Cx36 may play a role as a protective factor, for Cx36+ retinal cells had a greater increase in survival after injury than in Cx36- mice (Striedinger, et al., 2005).

Recently, scientists have been able to knock out (KO) the Cx36 gene in mice. Rodents without Cx36 have no blatant change in behavior or morphology compared to normal rodents (Maier, et al., 2002). They experience retinal and reproductive function loss but no change in motor function (Bennett & Zukin, 2004). To compensate for Cx36 loss, individual neurons change cytologically and electrophysiologically rather than make new gap junctions from other connexins (De Zeeuw, et al., 2003). No research has been done to see how Cx36 KO mice react to alcohol administration.

Cerebellum and Cx36

It has been shown that the cerebellum regulates movement timing as well as initiation and termination of motion (Bo, Block, Clark, & Bastian, 2008). The cerebellum fine-tunes movement through comparing actual and intended motion (Werner, Bock, & Timmann, 2008). The inferior olive of the cerebellum and its connection to climbing fibers regulate motor timing (Liu, Xu, Ashe, & Bushara, 2008). Animals with cerebellar mutations suffer severe ataxia (Kamens & Crabbe, 2007). Much of the ataxia experienced because of EtOH intoxication is expected to be from EtOH affecting the $GABA_A$ receptors in the cerebellum (Criswell, Ming, Kelm, & Breese, 2008). Excessive alcohol use causes a decrease in cerebellar mass (Oscar-Berman & Bowirrat, 2005). The specific behavioral changes experienced after acute alcohol intoxication are impaired coordination and gait, implicating alcohol action at the cerebellum (Belmeguenai, Botta, Weber, Carta, & De Ruiter, 2008).

Cx36 was found in the molecular layer of the human cerebellum (Belluardo, et al., 1999). It has been postulated that GJ synchrony within the cerebellum provides for motor function stabilization through feedback loops (Stoodley & Schmahmann, 2008; Van Der Giessen, et al., 2008). Many researchers have shown that Cx36 GJs are responsible for Purkinje cell synchrony (Long, Deans, Paul, & Connors, 2002; Placantonakis, Bukovsky, Aicher, Kiem, & Welsh, 2006; Van Der Giessen, et al., 2008), while some have shown that GJs are not a factor (Kistler, et al., 2002). Cx36 knock-out mice show no signs of ataxia, with normal performance on an accelerating rotarod and normal walking (Kistler, et al., 2002). In a recent study, it was found that Cx36 KO mice possess the same motor

performance as wild type (WT) mice but have impaired motor learning ability (Van Der Giessen, et al., 2008).

HYPOTHESES

It has previously been demonstrated in the Steffensen lab that acute EtOH suppresses the firing rate of DA neurons (Gallegos, et al., 1999) and Cx36-dependent ICPSDs (Stobbs, et al., 2004) of VTA GABA neurons in rats. This inhibition occurs at EtOH concentrations one-tenth of that known to directly enhance DA activity. Cx36 is expressed by GABA neurons in the VTA (Lassen, et al., 2007). EtOH, as well as quinidine, a non-selective Cx36 GJ antagonist, and mefloquine, a selective Cx36 GJ antagonist, suppress electrical coupling between VTA GABA neurons (Stobbs, et al., 2004). Given this evidence suggesting a role for Cx36 GJs in mediating EtOH effects on VTA GABA neurons, I hypothesized that mice lacking Cx36 would be less sensitive to the intoxicating effects of EtOH on behavior and VTA GABA neuron activity. To elaborate, I suggested that EtOH normally prevents electrical synaptic activity through these Cx36 GJs located in VTA GABA neurons, thereby disinhibiting DA neurons to increase dopamine release. Cx36 KO mice do not have the Cx36 GJs for EtOH to act upon and thus cannot cause its disinhibition of DA neurons.

Since the Steffensen lab had not previously studied VTA GABA neuron responses in wild-type (WT) mice, but their brain anatomy and physiology appear to be similar, I predicted that I will see similar effects of EtOH on VTA GABA neuron

excitability in WT mice as previously seen in rats. Moreover, I predicted that Cx36 KO mice would be insensitive to the intoxicating (i.e., ataxic) properties of EtOH and that they would not self-administer EtOH, or that EtOH self-administration of EtOH would be significantly less than that of WT mice.

METHODS

Animal Procedures

Male C57BL/6J (black) and CD-1 (albino) mice were bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Once weaned at PND 25, they were housed in maximum groups of four and given *ad libitum* access to solid food and water and placed on a reverse light/dark cycle with lights on from 8 PM to 8 AM. Mice were then housed separately after all EtOH injection and other drug testing to accommodate proper measurement of alcohol self-administration. Mice were given *ad libitum* access to solid food and water except during testing periods.

Drugs

Ethanol

On three consecutive days, while under isoflurane anesthesia, mice were injected intraperitoneally with 16% ethanol (EtOH), then subjected to behavioral tests after

recovery from anesthesia. We tested four doses of EtOH (0.75-4.0 g/kg) in random order of dosage separated by at least one day between injections. 0.75 g/kg EtOH is considered to be a mildly intoxicating dose whereas 4.0 g/kg is severely intoxicating. During self-administration drinking tests, mice were given 20% EtOH *ad libitum*.

Pentobarbital

After all EtOH injection and drinking experiments, mice were injected intraperitoneally with 25 mg/kg Pentobarbital and then subjected to behavioral tests. Pentobarbital is a GABA agonist.

MK-801

At least two days after Pentobarbital injections, mice were injected intraperitoneally with 0.5 mg/kg Mk-801 (dizocilpine). MK-801 is an NMDA antagonist.

Methylphenidate

At least two days after MK-801 injections, mice were injected intraperitoneally with 20 mg/kg Methylphenidate (MPH, Ritalin). Testing was delayed for 15 minutes to compensate for the delay in onset of MPH's effects. MPH inhibits the Dopamine Transporter (DAT), which blocks the reuptake of dopamine and norepinephrine from the synaptic cleft.

Mefloquine

Mefloquine, typically used as an antimalarial drug, is a slow-onset Cx36 GJ-specific blocker (Cruikshank, et al., 2004). CD1 male mice were injected with 40 mg/kg

of Mefloquine three days before testing. Control CD1 male mice were injected with an equi-volumetric amount of vehicle.

Open Field Assay for Motor Activity

To measure gross motor activity, WT to KO mice were compared in one of four open field chambers, each equipped with a piezoelectric transducer cemented to the underside of the suspended floor. The signal from the piezoelectric transducer was amplified 10X and filtered from 0.1-100 Hz (-3dB). It is well-known that mice habituate to their environment, becoming less active after a period of intense exploratory activity. A normal mouse will have elevated motor activity when first put in the chamber with a diminution in activity over a 30 min session. Piezoelectric activity is not only sensitive to locomotor activity but the frequency information inherent within the data enables evaluation of any rhythmic, tremor, or seizure activity as well. Locomotor activity was evaluated for 25 minutes after an injection of varying doses of acute EtOH, pentobarbital, MK-801, and ritalin in Cx36 KO mice and wild-type littermates. The Voltage Root Mean Squared (Vrms) indicates the amount of locomotor activity. These methods were repeated on CD1 mice injected with mefloquine and vehicle for EtOH testing as a control for the KO and WT mice.

Fixed Speed Rotarod for Balance Assay

To further study behavioral activity, the effects of EtOH on balance and motor coordination in Cx36 KO mice were evaluated. To accomplish this, the mice were tested on a fixed-speed rotarod, as previously described (Chandra, et al., 2008; Rustay, Wahlsten, & Crabbe, 2003). Mice were first trained on a rotarod until they were able to

remain on the rod for 300 seconds. Then mice were administered EtOH and replaced upon the rotarod to be measured for up to 300-second intervals at 30, 45, and 60 minutes after EtOH injection (the mice having been measured in the piezoelectric chambers for the first 25 minutes after injection). A 14 rpm rotarod speed and 300 second measurement interval remained the same through all experiments while injections varied in EtOH dose, pentobarbital, MK-801, or Ritalin. In the 300-second periods in which the mice are tested on the rotarod, the longest time the mouse could remain on the rod was recorded. The effect of time of all doses and drugs was compared between normal and Cx36 KO mice. These methods were repeated on mice injected with mefloquine and vehicle for EtOH testing as a control for the KO and WT mice.

Self-Administration

To determine if mice would self-administer EtOH, a drink-in-the-dark method was implemented (Rhodes, Best, Belknap, Finn, & Crabbe, 2005). As opposed to rats, mice will self-administer EtOH, so no sucrose fading procedure was necessary (Samson, Pfeffer, & Tolliver, 1988), nor is food deprivation. Three hours after the beginning of the dark cycle, both the KO and WT mice's normal water bottles were replaced by a sipper tube filled with 20% EtOH. Mice were then allowed to drink for two hours a day, for nine days. Alcohol drinking was measured using a graduated pipette sipper tube, recording the EtOH level before and after the two-hour period (Gupta, et al., 2008). Two thirds of the bedding was removed from the mouse cages before the drinking test to prevent potential bedding build-up that would cause sipper tube leakage. Bedding was replaced after the drinking test each day. These methods were repeated for five days on mice injected with mefloquine and vehicle as a control for the KO and WT mice.

To control for any physical characteristic that may affect a mouse's ability to drink (i.e., performance deficits), mice were given 10% sucrose for five days, two hours a day, to evaluate any significant differences between KO and WT mice (Gupta, et al., 2008). Any mice who were significantly low performers for sucrose drinking (i.e. more than two standard deviations away from the mean) were not included in the EtOH self-administration test.

Analysis

All analysis was ANOVA Single Factor, performed on Microsoft Excel. IGOR Pro was used in the creation of figures.

RESULTS

Open Field Assay for Motor Activity: EtOH Ataxia in Cx36 KO vs. WT Mice

The open field assay was used to measure the baseline motor activity as well as the ataxic, sedative or stimulatory effects of EtOH, pentobarbital, MK-801 and methylphenidate. Motor activity in the open field assay was evaluated for 25 min beginning 5 min after an EtOH injection. There was no significant difference in baseline motor activity between WT and KO mice as measured by the piezoelectric transducer ($P=0.74$, $F_{(1,14)}=0.12$; WT = 0.475 ± 0.04 Vrms, KO = 0.455 ± 0.04 Vrms; n=8,7 respectively). Intraperitoneal administration of EtOH (0.75-4.0 g/kg; **Fig. 2A**) significantly decreased motor activity in a dose-dependent manner in both WT mice (0.75 g/kg: $P=0.001$, $F_{(1,15)}=17.9$; 1.5 g/kg: $P=0.00098$, $F_{(1,14)}=17.884$; 2.5 g/kg: $P=0.0002$,

$F_{(1,14)}$=26.9; and 4.0 g/kg: P=4.7E-05, $F_{(1,14)}$=35.5; n=8 each) and KO mice (0.75 g/kg: P=0.000087, $F_{(1,13)}$=33.424; 1.5 g/kg: P=8.7E-05, $F_{(1,15)}$=33.4; 2.5 g/kg: P=3.7E-05, $F_{(1,13)}$=40.2; and 4.0 g/kg: P=3.0E-05, $F_{(1,12)}$=46). EtOH-induced motor ataxia was significantly greater in KO mice compared to WT mice (P=0.011, $F_{(1,55)}$=7.00).

Open Field Assay for Motor Activity: EtOH Effects in Mefloquine vs. Vehicle-treated Mice

As KO mice appeared to be more sensitive than WT mice to the ataxic effects of EtOH in the open field assay for motor activity, we tested the effects of IP administration of the Cx36 antagonist mefloquine (MEF) and vehicle (VEH) in WT mice in the open field assay to further evaluate the role of Cx36 gap junctions in EtOH ataxia. Intraperitoneal administration of EtOH (1.5-4.0 g/kg; **Fig. 2B**) significantly decreased motor activity in a dose-dependent manner, and EtOH-induced ataxia was significantly greater in MEF-treated compared to VEH-treated mice (P=0.016, $F_{(1,17)}$=7.2).

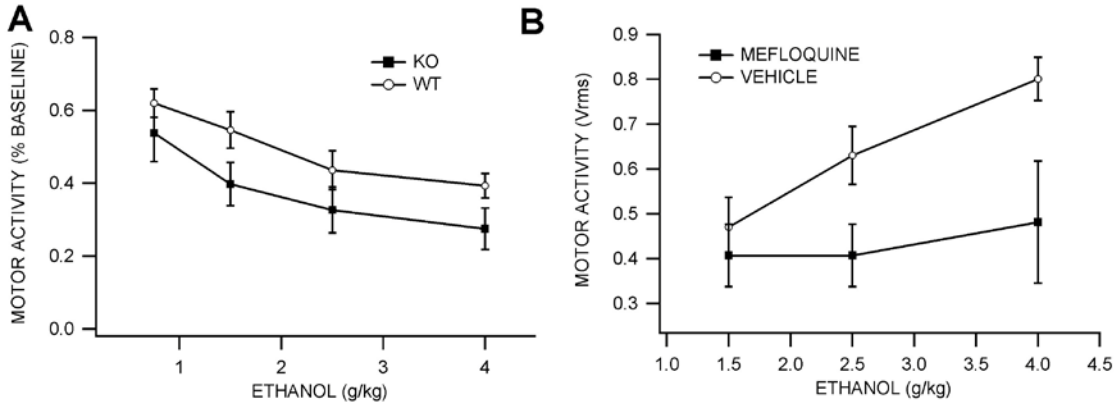

Figure 2. *Effects of acute intoxicating doses of EtOH on motor activity in KO vs. WT mice (A) and in mefloquine-treated vs. vehicle-treated mice (B).* EtOH-induced ataxia was significantly greater in KO than WT mice and in mefloquine-treated vs. vehicle-treated mice.

Open Field Assay for Motor Activity: Selected Drug Effects in Cx36 KO vs. WT Mice

As we have previously demonstrated differential effects of $GABA_A$ agonists and NMDA antagonists on Cx36 GJ-mediated electrical coupling between VTA GABA neurons (Stobbs, et al., 2004), we evaluated the effects of IP administration of the $GABA_A$ modulator pentobarbital and the NMDA antagonist MK-801 in KO mice compared to WT mice. Motor activity was measured for 25 min beginning 5 min after drug injection. Intraperitoneal administration of pentobarbital markedly reduced motor activity in both WT mice (**Fig. 3**; P=0.0002, $F_{(1,14)}$=27.3; n=8 each) and KO mice (P=2.7E-06, $F_{(1,14)}$=62; n=8 each). However, there was no significant difference in pentobarbital-induced ataxia between WT and KO mice (P=0.15, $F_{(1,14)}$=2.3). Intraperitoneal administration of MK-801 did not significantly reduce motor activity in either WT mice (**Fig. 3**; P=0.34, $F_{(1,14)}$=0.98; n=8 each) or KO mice (P=0.105, $F_{(1,123)}$=3.1; n=7 each). Moreover, there was no significant difference in MK-801 effects between WT and KO mice (P=0.33, $F_{(1,13)}$=1.02). Since pentobarbital and MK-801 ataxia were not affected in KO mice we tested the effects of MPH, an inhibitor of the DAT. Intraperitoneal administration of MPH significantly increased motor activity in WT mice (**Fig. 3**; P=0.002, $F_{(1,15)}$=13.8; n=8 each) but not KO mice (P=0.09, $F_{(1,13)}$=3.2; n=7 each) though there is a trend towards becoming significant from baseline that a higher sample size might reveal. There was no significant difference in MPH-induced ataxia between WT and KO mice (P=0.92, $F_{(1,14)}$=0.01).

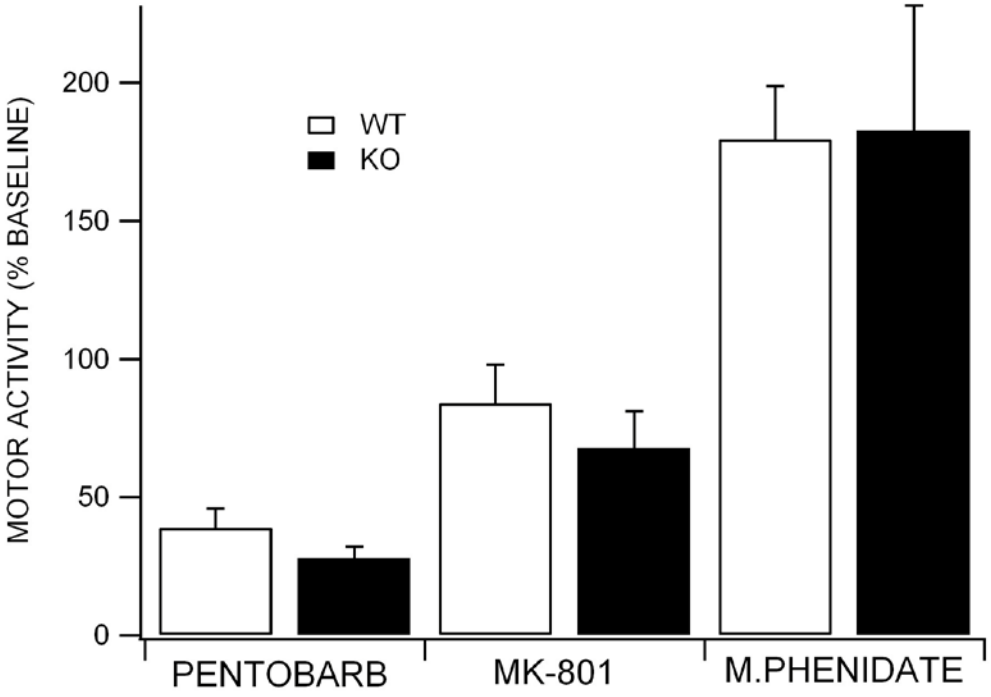

Figure 3. *Effects of acute intoxicating doses of pentobarbital, MK-801 and methylphenidate on motor activity in KO vs. WT mice*. The $GABA_A$ agonist pentobarbital markedly decreased motor activity in both WT and KO mice from baseline. The NMDA antagonist MK-801 did not significantly affect motor activity. The DAT inhibitor methylphenidate increased motor activity in WT mice. There was no difference in motor activity in WT vs. KO mice produced by these drugs.

Fixed Speed Rotarod: EtOH Ataxia in Cx36 KO vs. WT Mice

Following open field testing, the fixed speed rotarod apparatus was used to measure the ataxic (i.e., balance and coordination) effects of IP EtOH, pentobarbital, MK-801 and methylphenidate in WT vs KO mice. Although there was a tendency for KO mice to perform better on the rotarod, there was no significant difference in baseline rotarod performance between WT and KO mice following three days of training ($P=0.29$, $F_{(1,15)}=1.22$; WT = 265.13 ± 31.6 sec, KO = 300 ± 0.0 sec; n=8 each). Intraperitoneal administration of EtOH (0.75-4.0 g/kg; **Fig. 4A-D**) significantly decreased rotarod

performance in a dose-dependent manner at 30 min after injection in both WT mice (1.5 g/kg: P=.000002, $F_{(1,15)}$=60; 2.5 g/kg: P=1E-22, $F_{(1,15)}$=14996; and 4.0 g/kg: P=2E-19, $F_{(1,15)}$=5180; n=8 each) and KO mice (1.5 g/kg: P=0.00003, $F_{(1,15)}$=37; 2.5 g/kg: P=4E-18, $F_{(1,15)}$=3410; and 4.0 g/kg: P=5E-76, $F_{(1,15)}$=6.4E+11). However, when injected with 0.75 g/kg EtOH, WT mice have significantly decreased motor balance 30 minutes after injection compared to baseline but KO mice do not (WT: P=0.00072, F(1,13)=20.272; KO: P=0.0695, F(1,13)=3.972). There was a significant overall difference in the recovery from EtOH-induced ataxia in KO mice compared to WT mice, but only at the 0.75 g/kg dose level (P=0.0149, $F_{(1,41)}$=6.478) and 1.5 g/kg dose level (P=0.007, $F_{(1,47)}$=8.0) 30-60 min after injection, indicating faster recovery from ataxia.

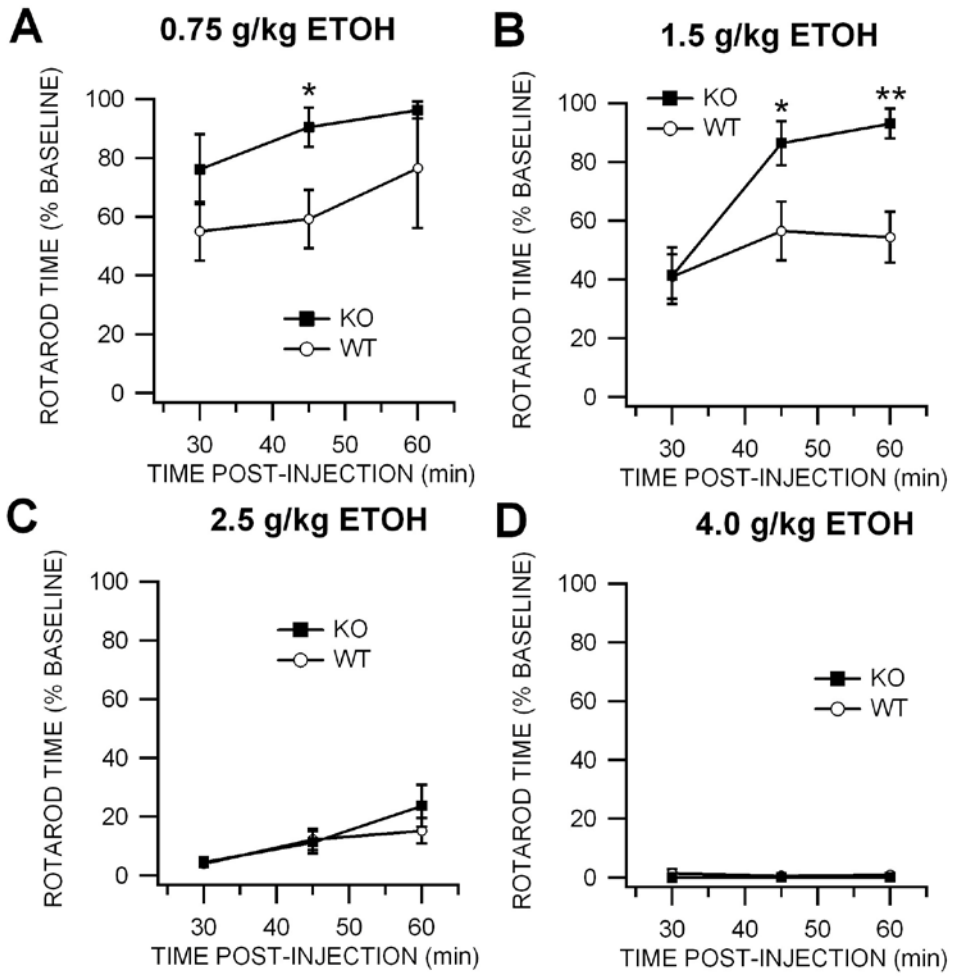

Figure 4. *Effects of acute intoxicating doses of EtOH on rotarod performance in KO vs. WT mice.* (A,B) At the 0.75 g/kg and 1.5 g/kg dose level, ETOH decreased performance in both WT and KO mice 30 min after EtOH injection.

However, there was a significant difference between WT and KO mice for the recovery from ETOH. (C,D) At the 2.5 g/kg and 4.0 g/kg dose levels there is even less rotarod performance produced by EtOH ataxia. Overall, KO mice recovered from ataxia sooner than WT mice at 0.75 and 1.5 g/kg EtOH. (Significance between WT and KO mice, *: P<0.05, **: P<0.01).

Fixed Speed Rotarod: EtOH Effects in Mefloquine vs. Vehicle-treated Mice

As KO mice appeared to be more sensitive to the ataxic effects of EtOH than WT mice, we tested the effects of IP administration of the Cx36 antagonist mefloquine (MEF) and vehicle (VEH) to CD1 mice in the fixed speed rotarod apparatus to further evaluate the effects of Cx36 GJs in ETOH ataxia. There was no significant difference in baseline rotarod performance between VEH-treated and MEF-treated mice tested three days after intraperitoneal injections of VEH and MEF and rotarod training (P=0.57, $F_{(1,7)}$=0.36; VEH = 275 ± 25 sec, MEF = 238.8 ± 54.8 sec; n=4 each). Intraperitoneal administration of EtOH (1.5-4.0 g/kg; **Fig. 5A-C**) significantly decreased rotarod performance in a dose-dependent manner at 30 min after injection in VEH-treated mice (1.5 g/kg: P=0.0035, $F_{(1,7)}$=22; 2.5 g/kg: P=9E-08, $F_{(1,7)}$=898; and 4.0 g/kg: P=1E-08, $F_{(1,7)}$=1831; n=4 each) and MEF-treated mice (1.5 g/kg: P=0.62, $F_{(1,15)}$=0.28; 2.5 g/kg: P=0.017, $F_{(1,7)}$=10.7; and 4.0 g/kg: P=7.1E-09, $F_{(1,7)}$=2112; n=4 each). Rotarod performance was significantly reduced by EtOH in MEF-treated mice compared to VEH-treated mice, but only at the 1.5 g/kg dose level at 30-60 min after injection (1.5 g/kg: P=0.049, $F_{(1,23)}$=4.33).

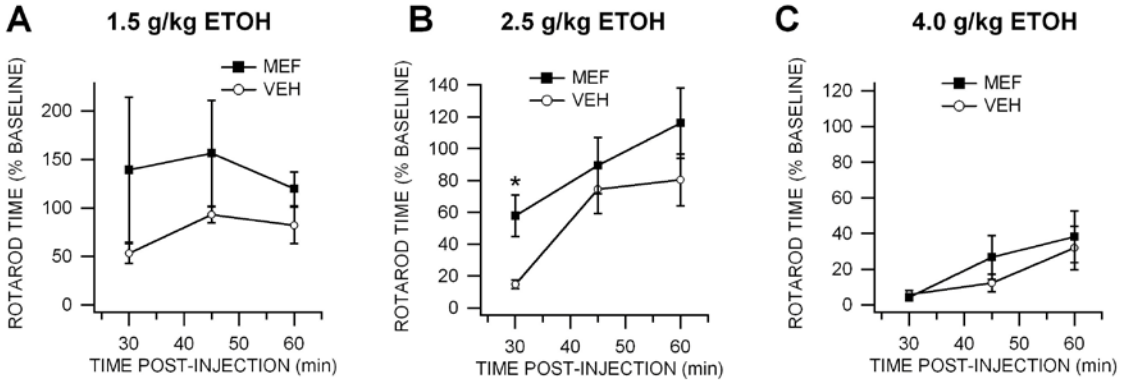

Figure 5. *Effects of acute intoxicating doses of EtOH on rotarod performance in mefloquine vs. vehicle-treated mice*. (A) At the 1.5 g/kg dose level, ETOH decreased performance in VEH-treated mice and MEF-treated mice 30 min after

EtOH injection. (B,C) At the 2.5 g/kg and 4.0 g/kg dose levels there is even less rotarod performance produced by EtOH ataxia. (Significance between WT and KO mice, *: P<0.05).

Fixed Speed Rotarod: Selected Drug Effects in Cx36 KO vs. WT Mice

As has been previously demonstrated, differential effects of $GABA_A$ agonists and NMDA antagonists on Cx36 GJ-mediated electrical coupling between VTA GABA neurons (Stobbs, et al., 2004) KO mice, we evaluated the effects of IP administration of the $GABA_A$ modulator pentobarbital and the NMDA antagonist MK-801 in KO mice compared to WT mice. Intraperitoneal administration of pentobarbital produced a marked decrement in rotarod performance at 30 min after injection in both WT mice (**Fig. 6A**; P=1.7E-20, $F_{(1,15)}$=7461; n=8 each) and KO mice (P=7.3E-09, $F_{(1,15)}$=201; n=8 each). However, there was no significant difference in pentobarbital-induced ataxia between WT and KO mice at 30-60 min after injection (P=0.48, $F_{(1,44)}$=0.513). Intraperitoneal administration of MK-801 produced a marked decrement in rotarod performance at 30 min after injection in both WT mice (**Fig. 6B**; P=5E-76, $F_{(1,15)}$=6.4E11; n=8 each) and KO mice (P=4.9E-65, $F_{(1,13)}$=4.9E11; n=7 each). However, there was no significant difference in MK-801-induced ataxia between WT and KO mice at 30-60 min after injection (P=0.898, $F_{(1,43)}$=0.018). Since ataxia produced by pentobarbital and MK-801 was not affected in KO mice we tested the effects of methylphenidate, an inhibitor of the dopamine transporter (DAT). Intraperitoneal administration of methylphenidate markedly reduced rotarod performance in WT mice (**Fig. 6C**; P=8.2E-05, $F_{(1,15)}$=30; n=8 each) but not in KO mice (P=0.051, $F_{(1,13)}$=4.7; n=7 each). There was a significant difference in methylphenidate-induced ataxia between WT and KO mice at 45-75 min after injection (P=5.7E-06, $F_{(1,44)}$=26.8).

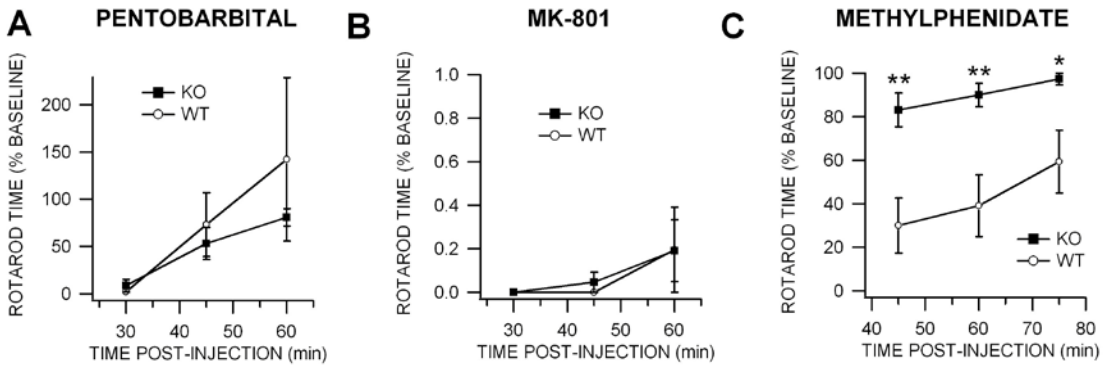

Figure 6. *Effects of acute intoxicating doses of pentobarbital, MK-801 and methylphenidate on rotarod performance in WT vs. KO mice*. (A) The $GABA_A$ agonist pentobarbital markedly decreased motor activity in both WT and KO mice. There was no significant difference in pentobarbital-induced ataxia between WT and KO mice. (B) While the NMDA antagonist MK-801 mildly affected open field motor activity (see Fig. 3) it significantly reduced rotarod performance. However, there was no significant difference in MK-801 ataxia between WT and KO mice. (C) While the DAT inhibitor methylphenidate increased open field activity (see Fig. 3) it had little effect on KO rotarod performance but significantly reduced it in WT mice. There was a significant difference in rotarod performance with methylphenidate between WT and KO mice. Note scale differences between A-C. (Significance between WT and KO mice, *: $P<0.05$, **: $P<0.01$).

Latent Differences in Rotarod Performance Between Cx36 KO and WT Mice

As mentioned previously, there was a tendency for KO mice to outperform WT mice in the rotarod test, albeit these results did not achieve significance. Thus, we evaluated the mice three weeks later in the fixed-speed rotarod to determine if there might be latent changes in performance. Indeed, WT mice had a decrement in performance compared to KO mice (**Fig. 7**; $P=0.001$, $F_{(1,14)}=17.9$)

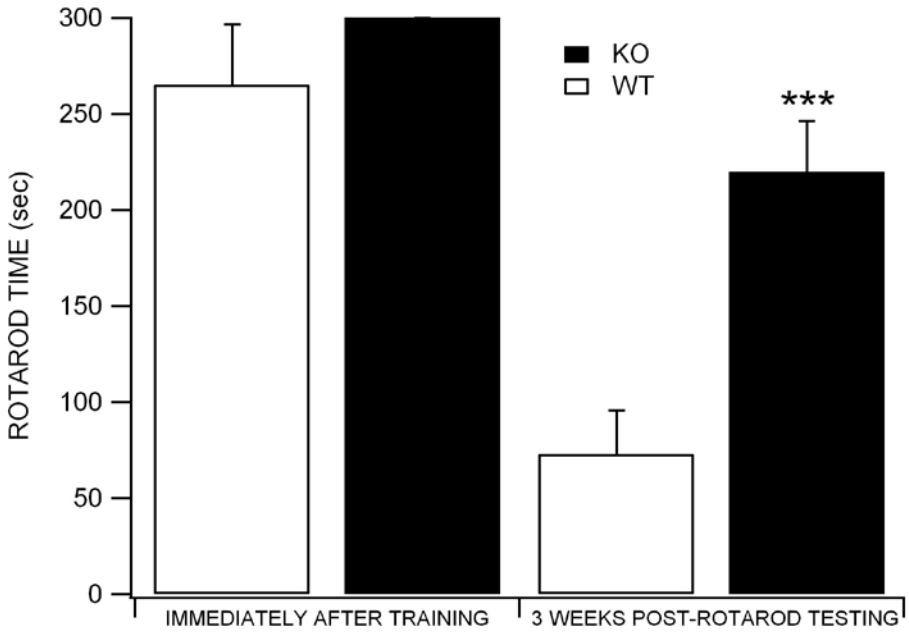

Figure 7. *Latent differences in rotarod performance in KO vs. WT mice*. While initial evaluation, immediately after training, of rotarod performance showed only slight differences between WT and KO mice, testing the mice in the rotarod three weeks after the completion of all rotarod testing revealed that KO mice performance in the rotarod was significantly greater than that of WT mice. (Significance between WT and KO mice, ***: P<0.001).

EtOH Consumption in Cx36 KO vs. WT Mice

As KO and MEF-treated mice appeared to be differentially affected by EtOH motor impairment compared to WT and VEH-treated mice, we sought to compare EtOH self-administration rates in these mice in order to evaluate the role of Cx36 GJs in EtOH seeking behavior. Compared to WT, KO mice consumed significantly less EtOH across the 9 days tested (**Fig. 8A**; P=9.2E-11, $F_{(1,125)}$=50.2). Similarly, compared to VEH-treated mice, MEF-treated CD1 mice consumed significantly less EtOH across the 5 days tested (**Fig. 8B**; P=0.003, $F_{(1,39)}$=10.3). In order to determine if there were any drinking performance deficits in KO vs. WT mice for EtOH consumption, we compared their consumption rates to sucrose. There was no significant differences in sucrose consumption rates between WT and KO mice (**Fig. 8C**; P=0.54, $F_{(1,69)}$=0.37). However, there was one KO mouse that exhibited pronounced deficit in sucrose consumption

(beyond two standard deviations of the mean). Thus, we excluded this mouse's EtOH self-administration data from the statistical analysis. It should be noted that the results would have been even more significant if this data was included.

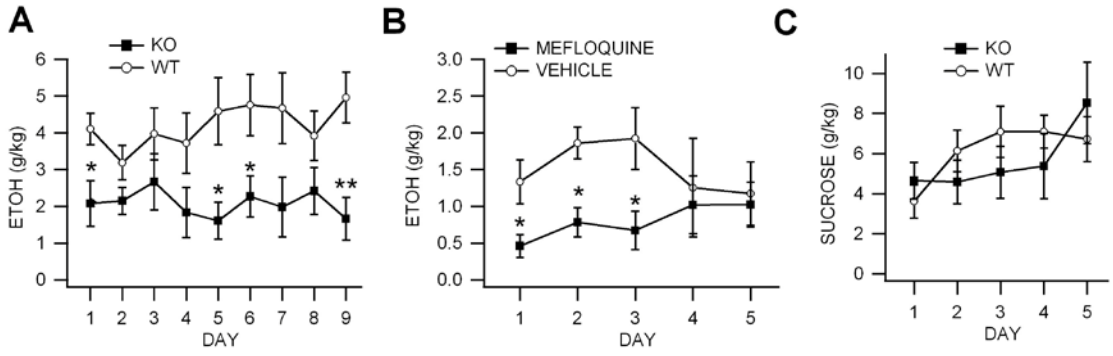

Figure 8. *Differences in self-administration of EtOH and sucrose in KO vs. WT mice and mefloquine vs. vehicle-treated mice*. (A) KO mice consumed significantly less EtOH than WT mice. (B) Mefloquine-treated mice also consumed significantly less EtOH than vehicle-treated mice. (C) There was no significant difference in sucrose consumption between KO and WT mice. (Significance between WT and KO mice, *: $P<0.05$, **: $P<0.01$).

DISCUSSION

I hypothesized that mice lacking Cx36 would be less sensitive to the intoxicating effects of EtOH on behavior and VTA GABA neuron activity. This was based on research suggesting that EtOH normally prevents electrical synaptic activity through these Cx36 GJs located in VTA GABAergic neurons, thereby disinhibiting DA neurons to increase DA release. Therefore, Cx36 KO mice do not have the Cx36 GJs for EtOH to act upon and thus cannot cause it's disinhibition of DA neurons. I predicted this would translate to Cx36 KO mice being insensitive to the intoxicating (i.e. ataxic) properties of

EtOH and that they would not self-administer EtOH. The results supported the latter prediction, but did not fully support the former.

KO mice self-administered significantly smaller amounts of 20% EtOH than WT mice (**Fig 8A)**. Similar findings resulted in the EtOH self-administration of CD1 mice injected with Mefloquine or Vehicle (**Fig 8B)**. This was not due to any physical incapability of KO mice to drink, for there was no significant difference in drinking 10% sucrose between KO and WT mice (**Fig 8C)**. A likely explanation is that KO mice do not experience as much pleasurable effects from EtOH as do WT mice. This supports the idea that EtOH normally inhibits Cx36 GJ in GABAergic neurons, thereby disinhibiting DAergic neurons, increasing DA release, causing euphoria. Since Cx36 KO mice do not possess these GJs, EtOH would not cause the change in inhibition on DA neurons. If there is no reward mechanism, there is no reason to expect excess drinking.

It is possible that the KO mice developmentally compensated for the lack of Cx36 GJs in order to regulate DA and other neurons typically controlled by VTA GABA neurons. It would seem if there is compensation in the physiology of reward, it is not affected by alcohol, or EtOH would cause a pleasurable sensation. KO mice likely already have an elevated basal level of DA because of lack of Cx36 GJs which decreases GABA neuron activity, whereby disinhibiting DA neurons. Mice injected with mefloquine are likely feeling the effect of raised DA levels even before the drinking test because of the mefloquine injection, blocking the Cx36 GJs. In fact, vivid dreams and hallucinations have been reported with mefloquine use (Juszczak & Swiergiel, 2009). EtOH would not have as potent an effect compared to the vehicle-injected mice, which have functioning Cx36 GJs.

The mefloquine-/vehicle-injected mice lose significance in EtOH drinking on the last two days of self-administration. Based on our previous studies with mefloquine and VTA GABA neurons (Allison, et al., 2006) it is possible that this could be due to mefloquine degrading and losing function, as mefloquine-induced inhibition of VTA GABA neuron ICPSDs was only evaluated up to 3 days after injection. It would be worthwhile to perform a longer self-administration test while giving maintenance mefloquine injections to the mice.

Most doses of EtOH significantly impaired both KO and WT's ability to run on the rotarod, with the exception of KO mouse motor activity at 0.75 g/kg (**Fig 4**). But EtOH only caused a significant difference between KO and WT rotarod times at the lower dose of 0.75 g/kg and 1.5 g/kg (**Fig 4A**). KO mice could run significantly longer than WT and recovered from ataxia faster. The results for mefloquine-/vehicle-injected mice were similar (**Fig 5**). They were also both significantly impaired by all doses of EtOH. The only difference between Mefloquine and vehicle-injected mice came at 1.5 g/kg EtOH (**Fig 5B**). To explain why only low doses of EtOH expose a balance difference between mice with and without Cx36 GJs, there are likely many molecular mechanisms regulating motor balance. Perhaps some of the other mechanisms besides Cx36 GJs, which both WT and KO mice possess, are only significantly affected by alcohol when there are large volumes of alcohol. This could be because there's a lower affinity of these proteins to alcohol so there needs to be more alcohol units to overcome the lower affinity.

Mice were also tested on the rotarod for developmental compensation of KO mice using the same drugs as were tested in the piezoelectric chamber (**Fig 6**). Pentobarbital

and MK-801 both caused a significant decrease in rotarod performance for both KO and WT mice (**Fig 6A-B**). However, there was no significant difference between KO and WT mice for either of the two drugs. MPH, though, caused a significant decrease of rotarod running time in WT mice (**Fig 6C**). Further, KO mice always performed significantly better on the rotarod than WT mice after being injected with MPH. Apparently, GABA neurons and NMDA receptors help to control motor balance but are not linked to Cx36 GJs. It also seems that the DAT normally plays a role in motor balance but that KO mice downregulate DAT as well so that balance is less affected when injected with a DAT inhibitor.

All doses of EtOH reduced locomotor activity of both WT and KO mice as measured in the piezoelectric chambers (**Fig 2A**). When examined dose by dose, there was only a significant difference between WT and KO mice when given 0.75 and 1.5 g/mg EtOH. However, gathering the data into an overall view of all EtOH doses, WT mice have much less gross motor impairment caused by EtOH than do KO mice. Apparently, Cx36 KO mice's gross motor capacity is more sensitive to the effects of alcohol. Results were replicated in Mefloquine-/vehicle-injected mice, which also evinced significantly lower movement activity in the mefloquine-treated mice than vehicle-injected mice (**Fig 2B**). This was an unexpected result, for I thought that because of the lack of Cx36 GJs, KO mice would be more resistant to EtOH's intoxicating effects than WT mice since there were no GJs upon which to act. Further, it is interesting that KO mice had a tendency to recover faster from alcohol-induced motor impairment on rotarod but had worse motor deficits in the piezoelectric chamber than WT mice. Perhaps the fact that the piezoelectric sensor is a measure of gross motor function,

whereas the rotarod is a more specialized by measuring balance function, can illuminate the reason behind the disparate results of the two behavioral tests (Crawley, 2007). The KO mice performed better on the rotarod which could mean that Cx36 GJs play a crucial role in regulating balance. But there may be many more factors regulating overall gross movement besides Cx36 GJs. Both WT and KO mice possess those factors so both have impaired gross movement when injected with EtOH. But the KO mice have gross motor deficits significantly greater than WT mice. It possible that KO mice have developmentally compensated for the lack of Cx36 GJs, as already discussed. As opposed to the reward compensation, however, the compensating mechanisms in the cerebellum *are* affected by alcohol, so that the KO have more gross motor deficits.

To test the possibility that Cx36 KO mice had developmentally compensated for the lack of Cx36 GJs, we applied a GABA agonist, pentobarbital, an NMDA antagonist, MK-801, and a DAT inhibitor, MPH (**Fig 3**). Pentobarbital significantly lowered and MPH significantly raised locomotor activity of both WT and KO mice but MK-801 did not significantly impact motor activity. While GABA neurons and the DAT play a role in locomotion, as well as Cx36 GJ (as shown in **Fig 2A**), GABA neurons and DAT do not compensate for Cx36 GJs in that role.

It is interesting to note that immediately after training on the rotarod, there is no significant difference in time between WT and KO mice (**Fig 7**). That is to be expected because they had just been trained to perform at a certain level equally. But when tested three weeks after the final use of the rotarod, KO performed significantly better than WT. This could either mean that KO mice, through some natural phenomenon caused by lack of Cx36 GJs, are naturally better performers on the rotarod and/or that they retain

working memory better than their WT counterparts. In all reports of Cx36 KO mice, nothing has been reported to have improved when compared to WT mice. In fact, it has only been noted that KO mice experience no motor change from WT (Bennett & Zukin, 2004). Even further, one study claimed that KO mice had merely "normal" rotarod performance (Kistler, et al., 2002), while another stated that KO mice had an impaired motor learning capacity (Van Der Giessen, et al., 2008). My data warrants a further look at KO mice's balance capacity and the ability to access memory. Cx36 GJ must have far ranging functions beyond those affected by alcohol.

Also, the fact that Cx36 KO mice seem "naturally" good at running on the rotarod could imply that perhaps they are better than WT at performing on the rotarod after an injection. That may seem unlikely because of the Pentobarbital and MK-801 results, when the KO mice performed just as poorly as WT mice.

While reflecting upon the quality of these tests, there were a few factors limiting our success and several aspects upon which we could improve. Cx36 KO mice experience reproduction function impairments (M. V. L. Bennett & Zukin, 2004), so it took longer than expected to breed new test subjects. I also received WT mice much later than the KO mice. While the WT mice do not have as much trouble reproducing, I had a late start in breeding a colony. If I had been able to breed more mice, I could have tested more subjects, which would help statistically. Also, because we did not have enough subjects to spare, I needed to use a different strain of mice (CD1) for the control mefloquine and vehicle injections. It would have been a better control to use WT mice for the injections. In addition, one Cx36 KO mouse died after all EtOH injection tests, before the self-administration tests, which again lowered our number of subjects. The

mouse died from an infection, not due to our testing. Finally, one of the four piezoelectric chambers would occasionally not work properly. This caused me to lose some data so there are again fewer subjects for the open field assay.

There are many worthwhile results in this study that deserve to be published for the benefits of other scientists. A few more studies ought to be completed. My lab is planning to further electrophysiologically study the difference in DAT levels between KO and WT mice to expand upon the differences found on rotarod performance. Also, if it were accomplishable to study ICPSDs in KO and WT mice, that could reveal much information. However, many early attempts proved fruitless. Nonetheless, the effort should continue because of the results' valuable potential. Finally, I need to find look at other compensatory mechanisms of the KO mice to reveal why there were differences between KO and WT mice in the behavioral and reward tests. There is valuable information here that can lead to better addiction treatment.

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