Ethanol Tolerance in the Rat Neurohypophysis: a Dissertation

Thomas K. Knott
University of Massachusetts Medical School

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ETHANOL TOLERANCE IN THE RAT
NEUROHYPOPHYSIS

A Dissertation Presented

By

Thomas Kris Knott

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
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In partial fulfillment of the requirements for the degree of:

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Thomas Kris Knott

Approved as to style and content by:

Dr. Jose Lemos, Committee Chairperson

Dr. Harvey Florman, Committee Member

Dr. Craig Ferris, Committee Member

Dr. Gyonghi Szabo, Committee Member

Dr. Gang Wang, Committee Member

Dr. Steven N. Treistman, Dissertation Advisor

Dr. Thomas B. Miller, Dean of Graduate School of Biomedical Sciences

Department of Biochemistry and Molecular Pharmacology, Neuroscience Program

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To Beth, whose love and belief in me made this all possible
Abstract

One of the main components underlying drug addiction is the emergence of tolerance. Although its development is a complex issue, and is believed to have both psychological and physiological connotations, it is clear that some physiological change must occur that would enable an organism to withstand drug concentrations lethal to a naïve system. The purpose of this thesis was to identify and study a physiological mechanism, whose characteristics were altered due to chronic exposure to ethanol.

Vasopressin (AVP), whose primary function is to control water balance, release from the neurohypophysis is suppressed by an acute ethanol challenge. Therefore, I hypothesized; 1) that chronic ethanol exposure would reduce the normal suppression of AVP release during an acute ethanol challenge and 2) that the ion channels that are acutely sensitive to ethanol, involved in the control of AVP release, would exhibit a change in their ethanol sensitivity and characteristics.

To study the hypothesis, I utilized the neurohypophysis from rats chronically exposed to ethanol and yoked controls to determine whether chronic exposure would modify the acute ethanol sensitivity of the neurohypophysial vasopressin release mechanism. I examined whether the long-term ethanol exposure affected the suppression of vasopressin release from either or both the intact neurohypophysis and the isolated neurohypophysial terminals. In addition, I investigated how chronic exposure affected two types of potassium channels, the ethanol sensitive large conductance Ca$^{++}$-activated (BK) channel
and the fast inactivating (I_A) channel known to be insensitive to physiologically relevant concentrations of ethanol.

I was able to establish that chronic ethanol exposure reduced the suppression of vasopressin release by an acute ethanol challenge from both the intact neurohypophysis and the isolated neurohypophysial terminals. In addition, I discovered that oxytocin release was affected similarly. I concluded from this data that chronic exposure to ethanol affected a general mechanism, which controlled hormone release from the neurohypophysis, and that this mechanism could be isolated to the neurohypophysial terminals.

I also used electrophysiological techniques to study ion channel characteristics of both the BK and I_A potassium channels. I found that in naïve rats, BK channels were potentiated and I_A channels insensitive to physiological relevant concentrations of ethanol. But in chronic ethanol-exposed rats the BK channels exhibited a reduced sensitivity to ethanol while I_A channels were inhibited. In addition, the current density of the BK channel was significantly reduced. These results show that at least one characteristic of each potassium channel has been modified. This suggests that chronic exposure can not only modify the ethanol sensitivity of ion channels known to be ethanol-sensitive, but also those believed to be relatively insensitive. Therefore, since modifications in these channels have previously been shown to alter the duration and frequency of action potentials, I conclude that these ethanol-induced modifications play a role in the modified hormone release patterns observed in the chronically exposed rats.
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Chapter 1

Introduction

to

Ethanol Tolerance in the Neurohypophysis
TOLERANCE

The phenomenon of tolerance to ethanol, after chronic exposure, is a complex issue and in the past was believed to have its roots in Pavlovian conditioning (Le et al., 1979; Wenger et al., 1981). Since then this psychological focus has begun to shift to identifying physiological changes or adaptations that occur following long-term exposure to ethanol. Although advances have been made, there is much unknown about the various adaptations in mammalian physiology that create organisms tolerant to blood alcohol levels that would be lethal to a naive system.

Tolerance can develop in a variety of forms and the type that occurs appears to be related to the duration of exposure to the drug (Kalant, 1998). Short-term or single exposures are believed to produce a brief period of drug insensitivity, known as acute or rapid tolerance. The acute form can be defined as a quick desensitization to the drug occurring within minutes during a continual exposure, that disappears within 24 hours without any negative physical after-effects (Leblanc, 1975). Rapid tolerance develops when a second exposure to a drug is received within 24 hours of the first. This form of tolerance also disappears without any negative consequences (Bitran, 1991). In contrast, repeated long-term exposure results in the development of chronic tolerance to the drug, that when withdrawn induces the onset of a variety of physical stressors, that in the worst cases can result in convulsions and death (Ros 1995; Crippen 2000). Thus, the development of tolerance to a drug following long-term exposure has been a major focus in the study of addiction and its causes.
Ethanol tolerance following chronic exposure has been a difficult area of study because of the many physiological alterations that occur at the cellular level. These modifications encompass an up-regulation of GABA$_A$ $\alpha$4, $\beta$2/3 and $\gamma$1 sub-units (Devaud et al., 1997), NMDA R1/R2A (Snell et al., 1996) and R2B sub-units (Follesa and Ticku, 1996), L-type calcium channels (Gerstin et al., 1998), and the down regulation of GABA$_A$ $\alpha$1 sub-units (Mhatre et al., 1993), neuropeptide Y receptors (Campbell and Erwin, 1993) and $IP_3$ receptors (Saito et al., 1996). In addition, there is also evidence of a reduction in adenylyl cyclase activity, an enhanced expression of inhibitory G-proteins (Wand et al., 1993) and Na$^+$/K$^+$ ATPase $\alpha$ sub-units (Chen et al., 1997), an increase in voltage-gated calcium current (Grant et al., 1993), and lipid alterations at the protein/lipid interface (Ho et al., 1994). The list of physiological changes due to chronic ethanol exposure is extensive, making it a formidable problem to address at the macroscopic level. Therefore, to better examine how chronic exposure to ethanol induces tolerance at the macroscopic level, a system already known to exhibit acute sensitivity to the drug should be examined.

THE NEUROHYPOPHYSIS AS A TOOL TO STUDY THE EFFECTS OF CHRONIC ETHANOL EXPOSURE

The neurohypophysis develops from neural ectoderm tissue and mainly consists of neural endings from cells, which originate in the supraoptic and paraventricular nuclei of the hypothalamus, and associated glia and blood vessels (Figure 1) (Hadley, 1992). These cells control the release of two hormones; vasopressin (AVP) and oxytocin (OT),
Figure 1. The neurohypophysial portal system for the release of vasopressin (AVP) and oxytocin (OT). Thanks to M.E. Hadley (1992).
into the bloodstream. Although these hormones are structurally similar and plasma concentrations of both are known to be reduced by ethanol (Eggleton, 1942; van Dyke and Ames, 1951; Hirvonen et al., 1966; Fuchs 1966), they serve quite distinct physiological roles. The primary function of AVP, also known as anti-diuretic hormone (ADH), is to maintain water balance while OT controls uterine contraction and milk release from mammary glands (Soloff et al., 1979).

It is well known that ethanol ingestion results in diuresis (Eggleton, 1942; van Dyke and Ames, 1951), which subsequently alters an organism’s water balance. Therefore, since ethanol affected water balance it was hypothesized by the Treistman and Lemos laboratories that ethanol would have an effect on AVP release (Wang et al., 1991a). Using a release stimulation protocol developed by Cazalis et al., (1987a), the group showed that AVP release from both the intact neurohypophysis and isolated neurohypophysial terminals was suppressed by acute ethanol challenges (Wang et al., 1991a, b). In addition, their research suggested a correlation between the inhibition of AVP release by ethanol with acute ethanol actions on calcium channels in the cell bodies and terminals of the AVP neurons (Wang et al., 1991a; Widmer et al., 1998). These findings have provided us with an optimal physiological system, proven to be affected by an acute ethanol challenge, in which to study the effect of chronic ethanol exposure.
Roles for potassium channels in hormone release

Action potentials (AP) are the driving force behind neurohypophysial hormone release via activation of voltage-gated calcium channels (Nordmann, 1983; Wang et al., 1993). Potassium channels are believed to modulate AP’s because they are involved in the afterhyperpolarization (AHP) of the membrane after the AP’s repolarizing phase and in addition, alteration of the channel’s activity affects spike frequency (Hotson and Prince, 1980; Wong and Prince, 1981, MacDermott and Weight, 1982). Plus, any variation in the efflux of potassium, such as either increasing or decreasing potassium ion flow, would hyperpolarize the membrane more quickly or slowly, respectively. Therefore, altering potassium ion flow during the repolarization of the membrane would effectively alter the period of time when voltage-gated calcium channels could be activated (Jackson et al., 1991), thereby, modifying the total amount of release following initiation of an AP.

POTASSIUM CHANNELS AS TOOLS TO STUDY THE ACUTE EFFECTS OF ETHANOL

Ion channels can be studied by measuring the ion flow, in the form of current, from one side of the membrane to the other through the channel pore (Hille, 1992). Via the use of a heat polished glass pipette, a high resistance gigaohm seal can be created between the tip of the pipette and a lipid membrane through which channel activity can
be recorded (Neher and Sakmann, 1976). Conventional whole-cell recordings are
obtained by rupturing the patch of membrane under the pipette, forming electrical
continuity between the pipette and cell interior, through which the total activity of a
channel population can be investigated (Hamill et al., 1981). An alternative form of
recording ion channel current is through the perforated patch technique. In this technique
an anti-fungal drug amphotericin B is added to the pipette solution (Rae et al., 1991).
This drug perforates cholesterol-containing membranes and provides continuity with the
interior of the cell through holes in the cell membrane in the patched portion of the cell,
large enough for only mono-valent ion flow. This method does not disrupt the internal
milieu of the cell and is therefore less invasive then conventional whole-cell recording.

**Calcium-activated potassium (BK) channels**

BK channels are a diverse class of channels (Marty and Neher, 1985), believed to
be composed of alpha and associated beta subunits, which are identified by their calcium
sensitivity and large channel conductance. Consistent with other voltage-gated channels,
BK channels are tetramers consisting of a channel pore composed of four alpha subunits
(Latorre, 1994). There are several closely related alpha subunits with differing calcium
sensitivity and gating characteristics which have been cloned from Drosophila
melanogaster (dSlo) (Adelman et al., 1992), mouse brain (mSlo) (Butler et al., 1993),
human brain (hSlo) (Pallanck and Ganetsky, 1994), and bovine aortic smooth muscle
(bSlo) (Moss et al., 1996). The alpha subunit is comprised of seven hydrophobic
transmembrane domains, and four additional hydrophobic domains, whose location
whether membrane bound or cytosolic, is in dispute (Figure 2). The known transmembrane segments are termed S0-S6, with segments S1-S6 having high homology with other voltage gated K⁺ channels. The S0 subunit is thought to interact with the accompanying beta subunit and play a role in channel modulation (Meera et al., 1997). The S4 transmembrane segment has a series of three positively charged arginines and is thought to play the role of a voltage sensor, similar to that in voltage-gated K⁺ channels (Jan and Jan, 1989). The four unlocalized hydrophobic regions on the carboxyl tail of the alpha subunit are known as S7-S10 and are believed to contain a calcium sensor between subunits S9 and S10 (Adelman et al., 1992; Bulter et al., 1993; Pallanck and Ganetsky, 1994, and Moss et al., 1996). Investigations with chimeric channels using mSlo and dSlo, which differ in calcium sensitivity, demonstrated that the degree of calcium sensitivity could be conferred by the carboxyl region (Wei et al., 1994). The beta subunits, of which there are four known types, are proposed to have two hydrophobic transmembrane domains and an extracellular loop (Figure 3) (Wallner et al., 1999). Investigators studying beta subunit substitutions have recently discovered that betas have the ability to alter activation kinetics (Brenner et al., 2000) and toxin-sensitivity (Xia et al., 1999) when associated with the alpha sub-unit. In addition, co-expression of the alpha and beta subunits in Xenopus oocytes has shown that the beta dramatically increases the calcium sensitivity of the alpha subunit (Meera et al., 1996).

Activation of this channel occurs during the repolarizing phase of the AP, and it is a main component in the repolarization of the membrane (Wang et al, 1992). In addition,
Figure 2. Eleven hydrophobic domains of the calcium-activated potassium channel (BK) alpha subunit. S0 is believed to interact with the beta subunit (Vergara et al., 1998), S4 contains the 3 positively charged arginine residues and the pore region lies between S5 and S6. In addition a calcium sensor is proposed to reside between S9-S10.
Figure 3. Two hydrophobic domains of the calcium-activated potassium channel (BK) beta subunit with an extracellular loop.
there are two types of this channel in the hypothalamo-pituitary axis, which differ in cellular location, calcium and drug sensitivity, and activation kinetics (Dopico et al., 1999), but have not been reported to have significant differences in ion flow.

**Ethanol effects on BK channels**

At clinically relevant concentrations ethanol has been shown to potentiate BK channel steady-state current in a dose-dependent manner (Madsen and Edeson, 1990). This effect is seen whether the channels are expressed in Xenopus oocytes (Dopico et al., 1998), reconstituted in a lipid bilayer (Chu et al., 1998) or in cloned pituitary (GH3) cells (Jakab et al., 1997). Our laboratory has studied ethanol’s actions on the neurohypophysial terminal BK channels using single-channel and conventional whole-recordings (Dopico et al., 1996). We learned that the ethanol induced increase in channel activity was due to a modification in channel gating and that ethanol had no effect on the channel’s conductance or voltage sensitivity. This finding was also observed in our lab’s investigations using cloned mSlo BK channels expressed in Xenopus oocytes (Dopico et al., 1998). However, the mSlo experiments demonstrated as well that an increase in cytosolic calcium concentration antagonized the ethanol effect. This suggests that when studying the effect of ethanol on BK channels, the calcium concentrations must be carefully regulated.
Fast-inactivating (I_A) potassium channels

Voltage-gated I_A potassium channels are found throughout the body and were first identified in isolated neural cell bodies of the marine gastropod Anisodoris (Connor and Stevens, 1971). They are a tetrameric structure composed of four identical alpha subunits that form a pore. The alpha subunit has six hydrophobic transmembrane helix domains labeled S1-S6 and a series of positive charges in the S4 helix, similar to the BK channel, which are thought to act as a voltage sensor (Figure 4). In addition, although voltage-gated K^+ (Kᵥ) channels have been reported to be associated with cytosolic beta subunits (Dolly and Parcej, 1996; Trimmer, 1998), which modulate activity by inducing rapid inactivation of otherwise non-Kᵥ channels (Heinemann et al., 1996; Sewing et al., 1996; Leicher et al., 1996; Gulbis et al., 1999), this association has not been established for I_A channels from neurohypophysial terminals.

The channel is inactivated when the membrane is at its resting potential and when activated, has a transient outward current carried by potassium ions that supply a substantial amount of the outward current seen in whole-cell neurohypophysial terminal recordings (Thorn et al., 1991). Activation of this channel begins membrane repolarization following initiation of an AP, with the current peaking within 10-25 ms. The current decays more slowly, but the majority of it dissipates within 25 ms after the peak is reached. In addition, the I_A channel can modulate the spike frequency and breadth of an AP (Bourque 1990; Thorn et al., 1991). Furthermore, when the channel is activated, potassium ions flow out of the cell, subsequently repolarizing the membrane.
Figure 4. Six hydrophobic domains of the fast-inactivating potassium channel (IA). S4 contains the charged arginine residues and the pore region lies between S5 and S6.
and enabling the cell to be restimulated. This process is an essential physiological phenomenon, since changing the state of membrane polarity would alter the amount of charge needed to reach the initiation threshold of an AP. Therefore, this channel plays a key role in release (Bondy et al., 1987).

**Ethanol effects on I\textsubscript{A} channels**

It has recently been reported by our laboratory that acute ethanol challenges in a physiological relevant range, 100 mM or less, have no effect on the peak current or inactivation kinetics of neurohypophysial I\textsubscript{A} channels from animals fed ad libitum (Wang et al., 1991a). These results are consistent with earlier reports of the effects of ethanol on I\textsubscript{A} channels from *Aplysia* neurons (Bergmann et al., 1974; Camacho-Nasi and Treistman, 1986; Treistman and Wilson, 1987). But these studies also showed that acute ethanol challenges above 200 mM on *Aplysia* metacerabral (MCC) and abdominal ganglion R15 cells, delayed the decay phase of the current. A delay in the onset of the I\textsubscript{A} decay phase would affect the time course of the AHP of the membrane following an AP by lengthening the spiking interval. In addition, although these ethanol concentrations are high, blood alcohol concentrations at these levels and greater are not uncommon in individuals being treated for alcohol toxicity in hospitals (Berild and Hasselbalch, 1981). Also, although the ethanol effects on the I\textsubscript{A} channel occur during exposure to high concentrations, it does not preclude the possibility that chronic exposure may alter
ethanol’s acute effect. Thus, the $I_A$ channel is a good candidate for a tool to investigate the possible modulation of inactivation kinetics following chronic ethanol exposure.

**SPECIFIC AIMS**

This thesis work characterized the effect that the development of tolerance, due to chronic ethanol exposure, has on hormone release and the potassium channels from the neurohypophysis. There were two primary aims, each with sub-categories, which were examined using hormone measurement and electrophysiology as methods of investigation.

First, the release of vasopressin from the neurohypophysis can be suppressed by an acute ethanol challenge (Wang et al., 1991a,b). This discovery lead me to hypothesize that chronic ethanol exposure would reduce the acute ethanol challenge’s suppression of AVP release. In studying this hypothesis I asked three questions; (1) Would a 1-2 hour exposure induce the same effect as a chronic exposure ?, (2) Would the effect be a general effect, also altering OT release ?, and (3) Is the environmental milieu of the intact neurohypophysis necessary or would an effect be observed using only the isolated neurohypophysial terminals ?

Second, chronic exposure to ethanol has been reported to have effects on channel populations (Gerstlin et al., 1998) and subunit composition (Mhatre et al., 1993; Snell et al., 1996; Follesa and Ticku, 1996; and Devaud et al., 1997). In addition, acute ethanol challenges have been shown to potentiate BK channel current in the neurohypophysis (Dopico et al., 1996). Therefore, I chose to examine the effect of chronic ethanol
exposure on the potassium channels of the neurohypophysis. My hypothesis was that chronic exposure would alter the population density and ethanol sensitivity of the channels. I studied this using conventional whole-cell recordings of channel currents before and during acute ethanol challenges and membrane capacitance measurements of isolated neurohypophysial terminals to calculate current densities.
Chapter 2

Tolerance to Acute Ethanol Inhibition of Peptide Hormone Release in the Isolated Neurohypophysis
Abstract

Acute ethanol (EtOH) exposure reduces the evoked release of vasopressin (AVP) and oxytocin (OT) from excised neurohypophyses and from dissociated neurohypophysial terminals of the rat. We show that rats placed on a diet which maintains blood levels of 30 mM EtOH for 20-40 days develop tolerance to acute EtOH inhibition of release. In the presence of 10 mM EtOH, high (50 mM) K⁺-induced release of AVP from isolated neurohypophysial terminals of EtOH-naïve rats was reduced by 77.7 ± 1.4%, while in the chronic EtOH group, release was reduced by only 9.4 ± 8.7%. Similar tolerance was evident during acute challenge with 75 mM EtOH, as well as for release of OT from isolated terminals. Animals treated with an intraperitoneal injection of EtOH, and sacrificed 90 minutes post injection did not exhibit the reduced EtOH inhibition of release from dissociated terminals during a 75 mM EtOH acute challenge. We conclude that the altered component(s) responsible for the tolerance to inhibition of release resides in the isolated terminal, since tolerance measured in vitro, from intact neurohypophyses was similar to that seen in isolated terminals. The failure of EtOH-injected animals to exhibit reduced inhibition of release in response to an acute EtOH challenge indicates that short-term elevated BAL does not induce this tolerance. The finding of tolerance to EtOH-induced inhibition of release from the intact neurohypophysis and isolated terminals provides a preparation in which to examine the molecular targets of acute drug action modified after chronic exposure to the drug.
Introduction

One of the more important aspects of drug addiction is the development of tolerance to the actions of the drug as a consequence of exposure. Various forms of tolerance have been reported, characterized by the time course of its development (Kalant, 1998). In this paper, we use the vasopressin (AVP) and oxytocin (OT)-releasing nerve terminals of the neurohypophysis as a model system to examine the development of tolerance to EtOH. The ingestion of EtOH has long been known to result in diuresis (Eggleton, 1942; van Dyke and Ames, 1951). Diuresis is controlled largely by the level of circulating vasopressin (AVP), which is released from terminals in the neurohypophysis, whose cell bodies lie in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) (Eisenhofer and Johnson, 1982). Ethanol has been shown to reduce circulating levels of AVP and OT (Kleeman et al., 1954; Gibbens and Chard, 1976; Eisenhofer and Johnson, 1982; Chiodera and Coiro, 1990), and suppress the release of AVP and OT from the hypothalamo-neurohypophysial system (Hashimoto et al., 1985; Wang et al., 1991a,b). In addition, chronic EtOH exposure reduces the number of AVP and OT-containing hypothalamic neurons (Kozlowski et al., 1989; 1990). Humans and animals develop tolerance to the acute diuretic actions of EtOH after chronic exposure to the drug (Schrier et al., 1979; Crabbe et al., 1981; Goldstein 1983; Pohorecky 1985).
The acute actions of EtOH on voltage-gated calcium channels, and calcium-activated potassium channels in both the cell bodies and terminals of AVP neurons have been correlated with inhibition of high K⁺-evoked peptide hormone release (Wang et al., 1991a,b; Widmer et al., 1998). In addition, the biophysical basis of EtOH’s actions on these channels has been described (Wang et al., 1994, Dopico et al., 1996). Thus, in addition to providing information on the cellular locus of drug tolerance, the hypothalamic-neurohypophysial neurons controlling peptide hormone release provide an ideal model system in which to determine whether the molecular targets of acute alcohol action are modified in response to chronic exposure. In this paper, we establish that the acute EtOH inhibition of AVP and OT release, from both the intact neurohypophysis and dissociated neurohypophysial terminals, is indeed, reduced after chronic exposure of the animal to the drug.

Methods

Animals: Male Sprague-Dawley rats weighing 250-300 g (Taconic Farms, Germantown, NY) were used in these studies.

Diet: Rats were maintained on a liquid diet (Research Diets, Inc., New Brunswick, NJ) containing 17 kcal% protein, 47 kcal% carbohydrate (Maltodextrin 42), and 36 kcal% fat. All liquids were supplied through the diet. When EtOH was added to the diet, it replaced 80% of the carbohydrate calories, and the diet was then composed of
17 kcal% protein, 11 kcal% carbohydrate (Maltodextrin 42), 36 kcal% carbohydrate (EtOH), and 36 kcal% fat. The alcohol-diet group was fed the control diet for two days, then the diet with 2.5% EtOH (w/v) for two days, followed by the diet with 5% EtOH (w/v). The alcohol-fed and control groups were fed on a staggered schedule to insure equal caloric intake; i.e., food intake in the EtOH-diet group (ad-lib) was monitored daily, and the yoked controls were fed an equivalent amount the following day. The eating and social behavior of the rats was monitored during 72 hours of continual videotape recordings.

**Hormone release experiments**

**Intact neurohypophysis:** After decapitation with guillotine, the intact neurohypophysis was isolated and washed in (mM): 145 NaCl, 5 KCl, 15 glucose, 1 MgCl₂, 2.2 CaCl₂, 10 HEPES, pH 7.3 (normal Lockes solution). The neurohypophysis was hooked onto a platinum wire in a plastic perfusion chamber (Cazalis et al., 1985), and perfused continually (125 μl/min). The perfusate was collected at two-minute intervals for peptide hormone determination. All solutions used in the perfusion were heated to 37° C. Neurohypophyses removed from EtOH-fed rats were in EtOH-free conditions for under 90 min., and the tissue may be considered to be in the initial stages of withdrawal when acute EtOH challenge is begun.
Isolated terminals: Neurohypophysial terminals were homogenized as previously described (Cazalis et al., 1987a) in a solution containing 270 mM sucrose, 10 mM HEPES, and 1 mM EGTA, pH 7.3. The homogenate was centrifuged at 100 g for 1 minute, the supernatant centrifuged at 2400 g for 3 minutes, and the pellet was resuspended in 1 ml of Lockes solution and distributed equally into four aliquots. The isolated terminals were loaded onto Acrodisc 0.45 μm pore size LC PVDF filters (Gelman Sciences, Ann Arbor, MI) and perfused.

Interperitoneal Injection: Ethanol-naïve rats were injected with a 16 % (w/v) EtOH solution in 0.9% saline (Bloom et al., 1982), resulting in a BAL of 34 ± 2.0 mM. A weight-matched set of controls was injected with a similar amount of solution consisting of only 0.9% saline. Both sets of animals were returned to their respective housing following the injections. After 90 minutes, the animals were sacrificed, measured for BAL, and their terminals were isolated for use in a release assay.

Hormone Release: Prior to hormone collection and assay, the terminals or intact neurohypophyses were rinsed with Lockes medium containing 0.02% (w/v) BSA for 45 minutes, followed by 0 Na⁺ Lockes (normal Lockes solution with an equimolar concentration of N-methyl-D-glucamine-chloride (NMDG-Cl) replacing the 145 mM Na⁺, (with 0.02% BSA) for 15 minutes. All buffer solutions were 305-310 mOsm. Depolarization-coupled release was stimulated with high K⁺ (50 mM) as described in Cazalis et al. (1987b). The concentration of NMDG-Cl was reduced when high K⁺ was used in the perfusion. Fractions were collected at 2 min intervals during the following sequence of solution changes: 0 Na⁺ Lockes (10 min); 0 Na⁺ Lockes containing 50 mM
K⁺ (4 min); 0 Na⁺ Lockes (20 min); 0 Na⁺ Lockes containing either 10 mM or 75 mM EtOH (4 min); 0 Na⁺ Lockes, 50 mM K⁺, and either 10 mM or 75 mM EtOH (matching the previous exposure) (4 min). Finally, fraction collection was continued during perfusion with 0 Na⁺ Lockes (20 min), followed by 0 Na⁺ Lockes containing 50 mM K⁺ (4 min), to determine possible hormone store depletion or residual EtOH effects. Basal release, determined by averaging the five fractions collected prior to each high K⁺ stimulation, was subtracted from the area under the curve (AUC) during 4 minutes of high K⁺ stimulation to calculate release in figures 3 through 6. The samples were frozen and stored at -80°C for quantitative analysis by radioimmunoassay or ELISA.

**Hormone assay:** Released hormones were measured by assaying 30 μl from every 250 μl fraction collected during each experiment with either an ELISA kit (Assay Designs, Inc.) or radioimmunoassay using 1²⁵ labeled AVP and OT (Wang et al., 1997). Values for AVP and OT were obtained in parallel from each collected fraction. The final antibody dilutions used in the radioimmunoassays were 1:120,000 for AVP and 1:90,000 for OT. The cross-reactivity of the OT antiserum for AVP was 0.015%, while that of the AVP antiserum for OT was 0.001%. The sensitivity limit of the assays was 0.5 pg for AVP and 1 pg for OT. In the ELISA assays, cross-reactivity of either antiserum for its opposing hormone was <0.001%, while sensitivity was 1.5 pg for AVP and 3 pg for OT. Individual samples assayed by both radioimmunoassay and ELISA yielded equivalent values of hormone concentrations.

**Blood alcohol levels.** To determine daily blood alcohol levels (BAL), 24 rats (3 groups of 8) were used in a blind study during the initial 25 days of the diet. The rats
were anaesthetized with halothane, and orbital-bleeds were collected into heparinized tubes. Samples were assayed for alcohol levels within 30 minutes. Animals used for orbital bleed measurements were not used for release studies. BAL levels for rats used in release assays were obtained from trunk blood collected in heparinized vacuum tubes after decapitation, and immediately centrifuged at 400g for 5 min to separate the serum from the plasma. The plasma was then stored at -20°C with minimal air in the tube. All EtOH assays were done using the Sigma Diagnostics 332-A NAD-ADH kit.

Chemicals: AVP antibody was graciously donated by Dr. R. John Bicknell of the AFRC Institute of Animal Physiology, Babraham, Cambridge, UK; OT antibody was graciously donated by Dr. Alan G. Robinson of UCLA Medical School, Los Angeles, CA; iodinated AVP and OT was from New England Nuclear (Boston, MA). Ethanol, Hepes, and MgCl₂ were obtained from American Bioanalytical (Natick, MA), NaCl and KCl were obtained from EM Science (Gibbstown, NJ), and CaCl₂ was obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis: All values in this study are reported as means ± SEM. The relationship between daily alcohol ingestion and blood alcohol level was evaluated using a Pearson’s correlation r-test. Differences between the naïve and chronic-EtOH exposed groups were evaluated using: Student’s t-test; one-way analysis of variance (ANOVA); two-way ANOVA; or three-way ANOVA. A post-hoc Tukey Honestly Significant Difference test was performed to evaluate multiple comparisons. Statistical significance for all analyses was set at $p < 0.05$. 

RESULTS

Blood alcohol levels (BAL), measured at the time of sacrifice in the EtOH-diet rats, was 31.4 ± 1.6 mM (Table 1), comparable to levels previously reported for rats on similar liquid diet protocols (Meert and Huysmans, 1994). In addition, studies using similar diet protocols reported little fluctuation in BAL during each 24 hour period (Meert and Huysmans, 1994; Ogilvie et al., 1997), consistent with our observation that alcohol intake was maintained throughout the day/night cycle (see below). Initial exposure to the EtOH-containing diet led to reduced food consumption, but food intake returned to levels observed prior to introduction of EtOH into the diet within two weeks. In spite of a steady increase in EtOH consumption (4.02 ± 0.15% daily increase in EtOH ingestion (g/kg) during 20 days), blood alcohol levels (BAL) peaked between day 9-13 of the diet, then declined to stable values of 30-35 mM by day 24 (Figure 5). The control group consumed essentially all of its food during the dark cycle, while the EtOH-exposed rats consumed their diet sporadically throughout both the light and dark cycle.

During the early phase of the conditioning trials (days 4-10) there was a positive correlation between BAL and alcohol consumption which was not evident during the latter stage of the conditioning trials (days 11-20) (Figure 5). This may reflect metabolic tolerance to the drug. Overall weight gain in the animals during the three-to-four week course of the diet protocol was similar in the EtOH-diet and the control-diet groups (Table 1). The overt behavior of the EtOH-fed rats was not qualitatively different from that of their yoked controls with respect to aggressive behavior towards conspecifics, or
<table>
<thead>
<tr>
<th></th>
<th>EtOH Naive</th>
<th>Chronic EtOH</th>
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<tr>
<td>Weight gain (%)</td>
<td>45.0 ± 3.3</td>
<td>42.4 ± 3.3</td>
</tr>
<tr>
<td>BAL (mM/L)</td>
<td>N.D. *</td>
<td>31.4 ± 1.6</td>
</tr>
<tr>
<td>AVP baseline release b,c</td>
<td>153.6 ± 90.6</td>
<td>167.0 ± 99.9</td>
</tr>
<tr>
<td>AVP baseline release w/EtOH b,d</td>
<td>145.4 ± 88.8</td>
<td>154.2 ± 76.2</td>
</tr>
<tr>
<td>OT baseline release b,c</td>
<td>115.4 ± 17.6</td>
<td>115.9 ± 18.1</td>
</tr>
<tr>
<td>OT baseline release w/EtOH b,d</td>
<td>118.9 ± 12.8</td>
<td>100.1 ± 10.7</td>
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* non-detectable. * pg/250 µl perfusate collection (2 minutes) from intact neurohypophyses (n=3). * average of 4 two minute collection points per animal (prior to ethanol addition). * average of 2 two minute collection points.
Figure 5. Blood alcohol level (BAL) and daily consumption of alcohol. Blood was drawn during the apex of the light (sleep) cycle between 12-1:00 PM. A Pearson's r-test comparing BAL and daily alcohol consumption from day 4 to day 20 indicated the absence of a linear relationship ($r(17) = 0.44 \ p > 0.05$) between the two parameters during this time span. The analysis was then separated into two segments, with the rising (day 4-10) and falling phases (day 11-20) in BAL analyzed separately. During the early phase of the conditioning trials (days 4-10) there was a positive correlation ($r(6)=0.84 \ p < 0.05$), between BAL and alcohol consumption indicating that BAL increased in parallel with alcohol consumption. During the latter stage of the conditioning trials (days 11-20), this relationship between BAL and alcohol consumption is lost ($r(10)=-0.24 \ p > 0.05$). Solid circles represent alcohol consumption ($n=23$) and hollow circles represent BAL ($n=6-12$). Gaussian best fit curves obtained using SigmaPlot 4.0 Jandel Scientific.
social behavior such as huddling and grooming, although the time spent in the latter interactions was reduced ~33% when compared to the yoked controls.

Baseline release of AVP and OT from intact neurohypophyses, from naïve and chronic animals, was measured in the absence and presence of EtOH (Table 1). There was no significant difference (i.e. p was > 0.05) in baseline release of AVP, with or without EtOH, in chronic or naïve animals. The same was true for baseline OT release, with or without EtOH, in chronic or naïve animals. Similar findings were observed from isolated terminals (data not presented).

Vasopressin release from isolated neurohypophysial terminals

In previous work, we have shown that release of AVP from a highly purified preparation of isolated neurohypophysial terminals is inhibited by acute EtOH challenge, in a similar manner to that seen in the intact neurohypophysis (Wang et al., 1991a). Chronic exposure of the rat to EtOH significantly reduced this acute suppression (Figure 6). Note that although the K+ stimulated release appears to be significantly greater in the naïve group shown in figure 6, this difference is not observed consistently as we have seen significantly greater K+ stimulated release in chronic animals during other experiments.

We next observed that K+ stimulated AVP release from nerve terminals from naïve rats, in the presence of 10 mM and 75 mM EtOH, was 22.3 ± 1.4% and
Figure 6. Representative example of AVP release, determined by RIA, from an isolated neurohypophysial terminal preparation, challenged with ethanol. Although the magnitude of hormone release during the initial high K⁺ stimulation differs in these two examples, this parameter did not consistently vary with diet type, and differences in the effects of ethanol challenge were independent of the magnitude of release during the initial depolarization. Data is representative of mean ± SEM of n=4 filters, refer to Methods section.
20.2 ± 2.3%, respectively, of values obtained in the absence of EtOH, while terminals from the chronic EtOH group, in the presence of the same EtOH concentrations, showed K⁺ stimulated AVP release of 90.6 ± 8.7% and 113.9 ± 15.2% of values obtained in the absence of EtOH (Figure 7). High-K⁺ evoked release of AVP from the isolated terminals of EtOH-naïve rats was not significantly different from release from chronically-exposed rats prior to EtOH challenge. In all cases, re-exposure to elevated K⁺ subsequent to measurements during acute EtOH challenge produced release values comparable to pre-EtOH challenge levels. Figure 2 shows the time-course of non-normalized release values, and also illustrates that baseline AVP release recorded prior to stimulation is similar in the EtOH-naive and the chronic EtOH groups. In addition, EtOH introduced prior to stimulation does not significantly alter the basal release of AVP (Table 1).

**Release after acute EtOH injection**

Removal of the neurohypophysis from the chronically-exposed animals occurs in the presence of circulating EtOH, whereas this is not the case for the naïve group. To assure that the reduction of acute inhibition is not simply the result of systemic EtOH at the time of sacrifice and dissection, four EtOH-naïve rats were injected interperitoneally with EtOH sufficient to raise the BAL to 34.0 ± 2.0 mM (Bloom *et al*, 1982). A matched set of controls was injected with saline and the effect of acute 75 mM EtOH challenge on AVP release from isolated terminals tested. A one-way ANOVA was performed to compare the EtOH-injected, sham-injected, EtOH-naïve-diet, and EtOH-diet (chronic)
Figure 7. AVP release from isolated neurohypophysial terminals during an acute ethanol challenge. A group-by-treatment ANOVA indicated that there was a significant difference in inhibition of release during acute ethanol challenge between chronic and naïve animals (F(1,16)=116.106, p<0.001), but not between 10 mM and 75 mM ethanol (F(1,16)=2.001) treatments, with no significant interaction between groups and treatments. RIA analysis was employed in experiments with 10 mM EtOH challenge and ELISA used in experiments with 75 mM challenge. Release_{EtOH} = K⁺-stimulated release in the presence of EtOH. Release_{Control} = K⁺-stimulated release in the absence of EtOH. Number of samples: chronic, 10 mM (n=6), naïve, 10 mM (n=8), chronic, 75 mM (n=3), and naïve, 75 mM (n=3).
groups. K$^+$ stimulated AVP release in sham-injected and EtOH-injected animals, during the acute EtOH challenge, was 32.0 ± 8.5% and 29.5 ± 6.7%, respectively, of values obtained in the absence of EtOH. Pairwise analysis indicated a significant difference between the chronic EtOH diet group and each of the other groups, whereas there were no significant differences between the values obtained with any of the other pairings (Figure 8). Thus, neither the presence of EtOH in the blood during sacrifice, nor the exposure of the animal to EtOH for 90 minutes is sufficient to cause the reduction of inhibition of release by 75 mM EtOH seen after prolonged exposure.

**Oxytocin Release from isolated neurohypophysial terminals**

The development of tolerance was also evident for the release of oxytocin from isolated terminals. K$^+$ stimulated OT release from terminals from naive rats, in the presence of 10 mM and 75 mM EtOH, was 19.8 ± 2.4% and 26.7 ± 4.6%, respectively, of values obtained in the absence of EtOH, while terminals from the chronic EtOH group showed K$^+$ stimulated OT release, in the presence of the same EtOH concentrations, of 91.3 ± 4.3% and 107.9 ± 14.9%, respectively, of values obtained in the absence of EtOH (Figure 9).
Figure 8. AVP release, determined by RIA, from naïve, sham-(0.9% saline) injected, ethanol-injected, and chronic ethanol diet animals following an acute challenge with 75 mM Ethanol. A one-way ANOVA analysis indicated that there was a significant group difference (F(3,22)=19.848, p<0.001). After adjusting α with a Tukey HSD, a posthoc q-test revealed that inhibition of AVP release in the chronic ethanol group was significantly different from that in all of the other groups (Q(3,22), p<0.001). There were no differences in other group comparisons (Q(3,22), p = (0.69-0.96)). Release_{EtOH} and Release_{Control} as described in fig. 3. Naive (n=3), sham injected (n=4), EtOH injected (n=4), and chronic (n=3).
Figure 9. Oxytocin release, determined by ELISA, from an isolated neurohypophysial terminal preparation during acute ethanol challenge. A group-by-treatment ANOVA indicated that there was a significant difference in inhibition of release between chronic and naïve animals (F(1,16)=42.331, p<0.001), but not between 10 mM and 75 mM ethanol (F(1,16)=1.001) treatments, with no significant interaction between groups and treatments. Release_{EtOH} and Release_{Control} as described in fig. 3. 10 mM (n=6), 75 mM (n=6).
Release from the intact neurohypophysis

We next examined whether the circuitry or environment within the intact neurohypophysis might alter the tolerance observed in isolated terminals. Release of both AVP and OT from intact neurohypophyses exhibited tolerance to acute EtOH challenge indistinguishable from that observed in isolated terminals. During acute challenge with 10 mM EtOH, K⁺ stimulated release of AVP and OT from naïve rats was 31.1 ± 14.8% and 25.1 ± 5.8%, respectively, of values obtained in the absence of EtOH, while in neurohypophyses from chronic animals, K⁺ stimulated release of AVP and OT during the same EtOH challenge was 90.7 ± 5.6% and 83.3 ± 15.4% of values obtained in the absence of EtOH (Figure 10). Comparison of release from the intact neurohypophyses and isolated neurohypophysial terminals indicated no significant differences in the suppression of AVP or OT release by the 10 mM acute EtOH challenge: 3-way ANOVA (F(1,28)=0.088, p=0.769).

Discussion

In vivo studies have shown that an acute challenge with EtOH reduces the AVP concentrations in human plasma during insulin-induced hypoglycemia (Chiodera and Coiro, 1990) or dehydration (Kleeman et al., 1954) and OT concentrations in women during spontaneous labor (Gibbens and Chard, 1976). In addition, there is evidence from whole animal studies that the acute reduction of circulating levels of these peptide
Figure 10. AVP and OT release, determined by RIA, from the intact neurohypophysis during acute challenge with 10 mM ethanol. A 3-way ANOVA indicated there was a significant difference in inhibition of release between the chronic and naïve groups (F(1,28)=151.194, \(p<0.001\)), but not between AVP and OT release (F(1,28)=0.525) or between intact neurohypophyses (n=3) and isolated neurohypophysial terminals (F(1,28)=0.088). There were no significant interactions between groups (F(1,28)<1.104 in all cases). Release_{EtOH} and Release_{Control} as described in fig. 3. Data for AVP release from isolated terminals are from figure 3, and data for OT release are from figure 5.
hormones produced by acute EtOH exhibits tolerance after chronic exposure to the drug (Crabbe et al., 1981; Schrier et al., 1979). Acute inhibition of peptide hormone release by EtOH can be observed in both the isolated intact neurohypophysis (Wang et al., 1991a), as well as in dissociated terminals (Wang et al., 1991b). In this paper, we show that tolerance to the inhibition of peptide hormone release by acute EtOH challenge also occurs in the excised, intact neurohypophysis (Fig. 10), as well as in dissociated terminals (Fig. 7 and Fig. 9), after chronic alcohol drinking in the rat. Tolerance was observed for both AVP and OT release. Although our preparation does not test the effects of chronic ethanol treatment on components of the AVP-OT system outside of the neurohypophysial nerve endings, such as cell bodies or osmoreceptors, these findings suggest that the circuitry and internal milieu found in the intact animal are not necessary for either the acute actions of the drug on release, or the demonstration of tolerance to this effect, after chronic exposure of the rat to the drug. It is not clear from this study, however, whether the isolated neurohypophysis would develop tolerance if exposed to EtOH for an extended period, or whether the intact animal is needed for the development of the phenomenon. In addition, though we did not use a group of ad lib fed animals in this study, previously published reports indicate that acute ethanol challenge also suppress the release of AVP in this group (Wang et al., 1991a,b). Nutritional deficits and a reduction in water intake during the early phase of the diet protocol may have effects on the AVP system, which would not occur in an ad lib diet animal. Although this aspect has not been investigated, reduced intake of food and water lasts only 5-7 days and once the animals
have been on the diet beyond 10 days, they exhibit weight gain similar to ad lib diet animals.

Under the conditions used in the experiments described in this paper, the degree of inhibition produced by acute EtOH challenge was similar for 10 mM and 75 mM EtOH. This agrees with previously-published data with isolated terminals, which showed only a marginal increase in EtOH-inhibition of release as concentrations are increased from 10 mM to higher values (Wang et al., 1991b). Tolerance may occur in varied forms, and on a number of time scales, from minutes to weeks (Kalant, 1998), and factors such as the duration of acute EtOH exposure preceding high K⁺-induced release may significantly influence the degree of inhibition. In addition, varied forms of tolerance may differ in their concentration-dependency. The apparent lack of concentration-dependency of the acute alcohol effect on release may reflect either a maximal effect at 10 mM concentrations, or may reflect the influence of short-term tolerance (on the scale of minutes), influencing the acute response in a concentration-dependent manner. Clearly, chronic drug exposure produces a shift in the EtOH-sensitivity of release, evident at both of the concentrations tested.

Recently, molecular targets for EtOH's acute actions on peptide hormone release from the neurohypophysis have been identified. Two membrane channels which play complementary roles in the rise in intracellular calcium required for peptide release in the nerve terminal are affected by the drug. L-type calcium channels are inhibited by EtOH (Wang et al., 1991a,b; Wang et al., 1994), and the BK subtype of calcium-activated potassium channel is potentiated by the drug (Dopico et al., 1996). Together, these
actions would effectively block the release process. The biophysical characterization of EtOH’s actions on these channels indicates that in both cases, it is the gating parameter which is altered by the drug, while other parameters such as voltage-dependency and ion selectivity are unaltered (Wang et al., 1994, Dopico et al., 1996). Moreover, when the BK channel is heterologously expressed from mRNA (Dopico et al., 1998) and even when BK channels are reconstituted into an artificial lipid planar bilayer (Chu et al., 1998), the actions of EtOH on the channel are similar to that seen in the terminal (Dopico et al., 1996). In addition, L-type calcium channels in the hypothalamic cell bodies of the neurohypophysial terminals, where spike patterns controlling release are generated, are also acutely inhibited by EtOH (Widmer et al., 1998). Thus, the present results, which indicate that tolerance to the actions of EtOH can be demonstrated at the level of the isolated neurohypophysial terminal, make this an ideal system in which to examine whether the mechanism for development of tolerance involves alterations of the channel proteins (or their microenvironment) which are known targets for the acute actions of the drug, and if so, the nature of these alterations.
Acknowledgement

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Chapter 3

Modifications in BK and I$_A$ potassium channel characteristics following long-term EtOH exposure
Abstract

Acute EtOH suppresses the release of vasopressin and oxytocin from the intact neurohypophysis and isolated terminals of the neurohypophysis. Our laboratory has demonstrated that the suppression of release is reduced in animals chronically exposed to EtOH, in both intact neurohypophyses and in isolated terminals (Knott et al., 2000). In this study, we examine whether the altered sensitivity of release is accompanied by changes in EtOH sensitivity or channel characteristics of the fast-inactivating (I_A) and calcium-activated (BK) potassium channels. Activity of the BK channel, in situ in the terminal, when expressed in oocytes, or when incorporated into planar bilayers, has been shown to be potentiated by acute EtOH. In Aplysia MCC and R15 cells, the decay phase of the I_A channel is delayed by EtOH. Two groups of rats were maintained on isocaloric diets, one of which had ethanol substituted for 36% of its carbohydrate calories for 20-40 days (blood alcohol levels plateaued at 30 mM). Whole-cell patch clamp recordings showed that BK currents from the chronically-exposed rats were significantly less sensitive to potentiation by acute EtOH, as shown by a rightward shift in the concentration-response curve, than were currents in terminals from yoked naïve animals. In addition, the current density of the BK channel from chronically-exposed animals is reduced. Also, the peak I_A current in the chronically-exposed but not the ethanol-naïve animals is inhibited in a dose-dependent manner by acute EtOH, but the inactivation kinetics remain unchanged.
Introduction

Tolerance, whereby the effects of a drug decrease as a consequence of exposure, represents a critical component of drug action, as well as a measure of neuronal plasticity. Various forms of tolerance have been described, characterized by the time frame, from minutes to weeks, in which they develop (Kalant, 1998). The molecular underpinnings of tolerance development are not yet understood. Typically, studies have utilized either preparations amenable to exploration at the molecular level for which the role of the molecules studied are not understood in terms of specific physiological or behavioral events, or a physiological or behavioral function is examined, for which the underlying molecular targets are unclear.

We have been using the rat hypothalamo-neurohypophysial system as a model to understand the acute and chronic actions of ethanol. This system provides a physiological readout of function, in the release of the peptide hormones vasopressin (AVP) and oxytocin (OT) from neurohypophysial terminals. The anatomic separation of cell bodies in the hypothalamus and terminals in the neurohypophysis offers a unique opportunity to examine independently, drug action in each of these compartments (Dopico et al., 1999).

It has been known for years that plasma vasopressin levels are decreased after ethanol ingestion (Eggleton, 1942; van Dyke and Ames, 1951; Hirvonen et al., 1966; Eisenhofer and Johnson, 1982), contributing to the diuretic response that follows a rise in blood ethanol. The diuretic effect of acute alcohol exhibits tolerance after prolonged ethanol exposure in both dogs and humans (Crabbe et al., 1981; Goldstein, 1983; Pohorecky,
1985; Schrier et al., 1979). We have previously shown that acute ethanol challenge blocks the release of AVP and OT from both the intact neurohypophysis and from isolated neurohypophysial terminals (Wang et al., 1991a,b). Recently, we have demonstrated tolerance to the acute inhibition of hormone release by ethanol after chronic ingestion of the drug, evident in both the intact neurohypophysis (Fig. 10) and isolated terminals (Fig. 7 and Fig. 9) from ethanol-fed rats (Knott et al., 2000).

The molecular machinery of release has been the focus of a large body of research (Hotson and Prince, 1980; Wong and Prince 1981; Bondy et al., 1987; Wang et al., 1991a,b; Catterall, 1999; Meir et al., 1999; Mochida, 2000; Lemos and Wang, 2000), providing us with a number of targets which might be expected to respond to acute ethanol, as well as exhibit plasticity in response to chronic drug exposure. Our previous studies have provided a biophysical description of acute ethanol effects on voltage- and calcium-gated channels in the hypothalamic magnocellular cell bodies and associated terminals (Dopico et al., 1996, Widmer et al., 1998). In this paper, we examine the changes (both in baseline characteristics and in sensitivity to ethanol) which occur in the calcium-activated potassium (BK) channel of isolated neurohypophysial terminals, as a result of chronic ethanol. For comparison, we also examine the transient potassium current (I_A) to question whether this potassium channel, not acutely sensitive to ethanol at intoxicating concentrations in the naïve rat (Wang et al., 1991a), exhibits plasticity after chronic exposure to the drug. Both of these channels affect spike characteristics and patterning to modulate peptide hormone release (Hotson and Prince, 1980; Wong and Prince 1981; Widmer et al., 1998; Wang et al, 2000 in press).
Methods

**Animals:** Male Sprague Dawley rats (Taconic Farms, Germantown, NY) weighing 250 to 300 g were used in these studies. Rats were maintained on a liquid diet (Research Diets, Inc., New Brunswick, NJ) as previously reported in chapter two.

**Preparation:** The neurohypophysis was removed and placed in low (~3 μM) calcium Lockes' solution (see solutions below), in which the pars intermedia was removed. Neurohypophysial terminals were isolated as previously described (Lemos and Nordmann, 1986). The dissociated terminals are placed in a sterile polystyrene dish filled with low calcium Lockes' solution for 3-4 minutes prior to perfusion with 2.2 mM calcium Lockes. This allows the terminals to form a loose attachment to the dish, but to be lifted off the bottom after a tight seal is formed. The dissociated terminals were 6-12 μm in diameter and easily identified using phase and interference (Hoffmann) optics.

**Whole-cell recordings:** BK currents were obtained from dissociated terminals using the whole-cell patch-clamp technique (Hamill et al., 1981). The data were acquired using an A/D converter and Pclamp6 software (Axon Instruments, Burlingame, CA). All amplitudes of the outward BK current were measured during the current plateau, 250-500 msec after the beginning of the voltage step. Currents were recorded using a patch-clamp amplifier (Axopatch 200B, Axon Instruments Inc., Foster City, CA) at a bandwidth of 5 kHz and leak-subtracted off-line.
Electrodes were pulled (David Kopf Instruments, Tujunga, CA) from 100 μl glass pipettes (Drummond Scientific Co., Broomall, PA). The electrode shanks were coated with Sylgard (Dow Corning Co., Midland, MI) to reduce capacitance, and the tips were fire-polished on a microforge (Narashige, Tokyo, Japan) to give a resistance of 4-8 MΩ when filled with pipette solution (see below).

Solutions: For conventional whole-cell recordings the pipette solution consisted of 120 mM K-Gluconate, 10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (Hepes), 20 mM N-methyl-D-glucamine-Cl, 15 mM KCl, 2.25 mM CaCl₂, 2.65 mM MgCl₂, 2 mM EGTA, 2 mM HEDTA, 0.2 mM cAMP, 2 mM Mg-ATP, pH 7.3 and 315 mOsm. Final free Ca²⁺ concentration ~ 4 μM (Fabiato, 1988). Calcium and HEDTA are omitted in the 0 calcium experiments. For perforated-patch technique the pipette solution consisted of 130 mM K-Gluconate, 20 mM KCl, 5 mM NaCl, 5 mM glucose, 2 mM CaCl₂, 325 μM Amphotericin B, pH 7.3 and 315 mOsm.

In all whole cell recordings the terminals are bathed in Lockes' solution which consisted of 130 mM NaCl, 15 mM glucose, 10 mM HEPES, 5 mM KCl, 2.2 mM CaCl₂, 2 mM MgCl₂, pH 7.3 and 305 mOsm. In low (3 μM) calcium Lockes' solution, the 2.2 mM CaCl₂ is reduced to 1.96 mM and 2 mM EGTA is added. For the 0-calcium experiments 5 mM BaCl₂ is used in place of 2.2 mM CaCl₂. An osmotic difference of 10 mOsm is maintained between the pipette and bath solutions to enhance seal formation.

Chemicals: Ethanol, HEPES, and MgCl₂ were obtained from American Bioanalytical (Natick, MA). BaCl₂ and CaCl₂ were from Fisher Scientific (Fair Lawn, NJ). K-gluconate, glucose, N-methyl-D-glucamine, HEDTA, EGTA, Tetraethyl-
ammonium chloride (TEA), 4-aminopyridine (4-AP), Amphotericin B, charybdotoxin, cAMP, and Mg-ATP were obtained from Sigma Chemical (St. Louis, MO). NaCl and KCl were from EM Science (Gibbstown, NJ).

**Statistical Analysis:** All values in this study are reported as means ± SEM. We evaluated the differences between the naïve and chronic-ethanol exposed groups using a Student’s t-test. Statistical significance for all analyses was set at $p < 0.05$.

**Results**

To investigate the effect of an acute ethanol challenge on the potassium channels of isolated neurohypophysial terminals, channel subtypes must first be identified using pharmacological methods. Whole-cell recordings and appropriate pharmacological blockers were used to isolate two predominant outward currents, the calcium-activated potassium (BK) current and the fast, transient A-current ($I_A$) in neurohypophysial terminals, as well as a residual non-potassium current (Fig.11).

Results obtained with acute ethanol exposure confirmed previous findings that BK current in the naïve animal terminal is augmented by intoxication levels of ethanol while $I_A$ current is unaffected (Fig 12a,c). Also, the residual non-potassium current is unaffected by an acute challenge with 100 mM EtOH (Fig. 12e). In addition, we provide evidence that after chronic exposure to ethanol, BK channels are less sensitive to acute potentiation and that the $I_A$ channel’s peak current is inhibited during an EtOH challenge (Fig 12b,d). To confirm that the ethanol potentiated current we had identified (Fig 12b,c)
Figure 11. Identification of three separate currents found in the isolated neurohypophysial terminal by their pharmacology. Traces in this and subsequent figures are the average of four repetitions of the following stimulus: the terminal membrane is clamped at -80 mV and stimulated with a voltage step to +40 mV for 500 msec. All drugs and external solutions are perfused directly onto terminals raised from the dish bottom. The upper trace is the total current, recorded using an external media of 2.2 mM calcium normal Locke's solution. Following a 7 mM application of the IA channel inhibitor 4-aminopyridine (4-AP), the fast inactivating component of the current is removed leaving a non-inactivating current middle trace. This current is partially blocked by 100 mM tetraethylammonium-chloride (TEA), a potassium channel inhibitor, leaving a residual current lower trace which is insensitive to the potassium channel blockers.
Figure 12. Sensitivity to 100 mM EtOH in naïve and chronic-ethanol exposed groups. The 4-AP sensitive $I_A$ current (A) naïve, (B) chronic. The non-inactivating potassium current which is sensitive to TEA but insensitive to 4-AP (C) naïve, (D) chronic. (E) The residual current insensitive to 4-AP and TEA. Black – control trace, Red – 100 mM EtOH, Blue – 2.2 m Calcium bath traces before and after EtOH treatments. The currents were isolated using the protocols from figure 11.
was the BK current which had previously been observed in the neurohypophysial terminals (Dopico et al., 1996a,b), we verified that the ethanol-potentiated current was calcium-dependent (Fig 13a) and that the reversal potential was that of a potassium channel (Fig 13b). These findings indicated that the ethanol-potentiated channel we were examining was the previously identified neurohypophysial calcium-activated potassium (BK) channel.

A concentration-response curve generated by acute exposure to varying concentrations of ethanol indicate that potentiation of BK currents is shifted along the concentration axis in a manner indicative of decreased sensitivity as a result of chronic exposure (Fig. 14). We also observed a dose-dependent inhibition of I_A current in neurohypophysial terminals from chronic animals, but no such sensitivity in the terminals from naïve animals (Fig. 15). We were not able to use concentrations above 150 mM for the I_A channel experiments because terminals from naïve animals have a small time frame of survival when exposed to concentrations this high and results are difficult to interpret. On the other hand, terminals from chronic animals survive quite well in concentrations up to 150 mM. Therefore, although there is not a complete dose response curve, the data indicates that I_A channels from naïve animals respond minimally, at most, to an acute ethanol challenge and those from the chronic animals are inhibited in a dose-dependent manner.

We next asked whether there were changes in BK baseline current characteristics as a result of chronic exposure. Such changes might provide insight into the basis for the shift in sensitivity, since different isoforms of the single slo gene generating the channel-
Figure 13. Characteristics of the 4-AP insensitive, TEA sensitive potassium channel A) Zero calcium eliminates the current which is potentiated by ethanol. Residual currents are subtracted and holding potential, step, and traces averaged as in figure 11. Black - 5 mM barium with 7 mM 4-aminopyridine, red - 50 mM ethanol. B) Tail-current analysis of reversal point for chronic and naïve groups. Traces collected during perfusion with 2.2 mM calcium Locke’s solution with 7 mM 4-aminopyridine. Holding potential -80 mV, step to +40 mV and down to potentials on x-axis. Residual current subtracted. (n=4). p = 0.32
Figure 14. Histogram of the dose dependent responses of BK channel current to a series of acute ethanol challenges. The channels were isolated using the protocol in figure 11. The membrane was clamped at -80 mV and stepped to +40 mV. Current measurements were averaged over the last 250 msec of each set of traces. Each group was exposed to 3 or more concentrations of ethanol. (n=3-4).

* p < 0.05
Figure 15. $I_A$ acute ethanol challenges. Acute ethanol challenge of naïve and chronic animals. Peak current measurement of the fast inactivating 4-AP sensitive current during the initial 50 ms of the trace. Currents isolated using the protocol in figure 11 (n=4).

* $p < 0.05$
containing alpha subunit, or association with one of the auxiliary beta subunits is known to alter parameters such as activation kinetics and Ca-sensitivity of the BK channel (Xia et al., 1999; Wallner et al., 1999; Brenner et al., 2000; Meera et al., 1996). Neither the activation kinetics (Fig. 16a) nor the voltage-dependency of the BK current (Fig. 16b) were altered as a result of chronic exposure.

In addition to the tolerance conferred by the shift in potentiation of BK channel activity by acute ethanol challenge, adaptive changes might include a reduction in the number of channels or conduction properties of the channels, to counteract the acute potentiation of current. Such changes have been noted for voltage-gated Ca channels and calcium transport, which are upregulated in response to chronic drug exposure in a number of preparations, countering the acute inhibition produced by the drug (Harris and Hood, 1980; Stokes and Harris, 1982; Pozos and Oakes, 1987; Gerstin et al., 1998). We used capacitance measurements to determine membrane area of the terminals, and then calculated the current density in naïve vs. chronically-exposed terminals. Capacitance was linearly related to the diameter of the terminal, and this relationship was unaltered by drug treatment, suggesting that infolding was not induced or lost (Fig. 17a). Current density was significantly reduced in terminals obtained from the rats on the alcohol diet (Fig. 17b). In addition, terminals from naïve and chronic animals showed no significant differences when comparing a capacitance to diameter ratio; naïve 0.148 ± 0.006, chronic 0.139 ± 0.007. Therefore, it is unlikely that the difference in density was related to an unbalanced sampling population.
Figure 16. BK channel characteristics. (A) Normalized current traces of the activation kinetics from naïve ($\tau = 1.046 \pm 0.290 \text{ ms, } n=7$) and chronic ($\tau = 1.152 \pm 0.196 \text{ ms, } n=8$) groups. Currents were isolated using protocols from figure 11. The single activation component of the BK channel was measured using pClamp6. The kinetics were evaluated over 20 msec beginning at the initiation of the channel’s activation. (B) Voltage dependency comparison from the naïve (solid line, filled circles) and chronic (dashed line, hollow circles) groups (n=4). There were no significant differences in either characteristic.
Figure 17. Current density comparison of BK channel current in isolated neurohypophysial terminals. (A) A group comparison of each terminal’s capacitance to its diameter. Naive (hollow), chronic (filled). (B) Current density comparison between naïve (n=12) and chronic animals (n=14). Currents measured and isolated as in protocol in figure 11. Capacitance was measured using the Axopatch 200B amplifier. * p<0.05.
We also asked whether there were any changes in the kinetics or current density of the $I_A$ channels from chronically-exposed animals. The two inactivation components of the $I_A$ current from naïve, chronic, or previously published reports on rats fed ad libitum (Thorn et al., 1991) were not significantly different (Fig 18a). In addition, there was no statistically significant change in channel density apparent for $I_A$ after chronic exposure, in contrast to the decreased density seen for BK current. (Fig. 18b).

**Discussion**

It has previously been demonstrated *in vivo* in the neurohypophysis and *in vitro* in the oocyte expression system that BK channels are sensitive to acute challenges with physiological relevant concentrations of ethanol (Dopico *et al.*, 1996a,b; Dopico *et al.*, 1998). These channels play a major role in shaping the action potential and are potentiated by ethanol concentrations as low as 10 mM. In contrast, the $I_A$ channel from neurohypophysial terminals, which also has a major role in control of the action potential, has not been shown to be affected by ethanol concentrations as high as 150 mM (Wang *et al.*, 1991a) and is therefore, in this preparation, considered to be insensitive to ethanol.

In the present report we present data showing that the ethanol sensitivity of both these channels has been altered due to chronic long-term exposure to the drug. The BK channel from the chronically exposed animal does not exhibit sensitivity to an acute ethanol challenge until concentrations are greater than 50 mM and when examined over a series of ethanol concentrations we discovered there was a shift in the dose-response
Figure 18. Comparison of $I_A$ channel characteristics. (A) Series of currents from ad libitum fed, naïve, and chronic animals showing no difference in their inactivation kinetics; ad libitum ($\tau = 36.35 \pm 8.58$ ms), naïve ($\tau = 35.56 \pm 3.20$ ms), and chronic ($\tau = 31.35 \pm 2.65$ ms).  
(B) Current density comparison between naïve ($n=7$) and chronic ($n=8$) groups. Currents measured and isolated as in protocol in figure 11. Capacitance was measured using the Axopatch 200B amplifier.
curve (Fig. 14). This is evidence that tolerance to a drug, following long-term exposure, has occurred. In addition, we have established that chronically exposed animals show a significant reduction in BK current density (Fig. 17). This result suggests that there could either be a reduction in the channel population or a change in the conduction properties of the channel. There is precedence for chronic exposure resulting in a channel population change, as studies on PC12 cells chronically exposed to ethanol show an increase in the L-type calcium channel population (Gernstin et al., 1998).

When we examined the effect of chronic exposure on the potassium $I_A$ channels, our findings showed that in naïve animals the channel was minimally effected by ethanol concentrations as high as 150 mM, similar to that previously observed in animals fed an ad libitum diet. In contrast, the $I_A$ channel from the chronically exposed animals expressed acute ethanol sensitivity beginning at 100 mM (Fig. 15). These results are the first evidence that a channel previously believed to be insensitive to a drug, had gained sensitivity following chronic exposure to the drug.

These two potassium channels are known to be involved in hyperpolarization of the membrane following neurotransmitter or hormone release (Lang and Ritchie, 1987). The $I_A$ channels are believed to activate during the rise of the action potential, initiating the beginning of membrane repolarization, and are followed by BK channel activation, which returns the membrane to its resting potential. In addition, inhibition of either BK or $I_A$ channels have been shown to broaden the action potential (Wang and Lemos, submitted). Therefore, any changes in the characteristics of the channels may result in altering the excitability of neurons and their capability to reach threshold to fire action
potentials. Thus, how the chronic effect of ethanol exposure on these two potassium channels plays a role in the CNS must be examined.

We have shown previously that the chronic animals maintain blood alcohol levels above 30 mM (Fig. 5 and Table 1). This concentration increases the probability of the BK channel from the neurohypophysis being open 2.5 times more than normal (Dopico et al., 1996a). Therefore, an animal’s physiology may respond to the continual EtOH exposure by reducing the channel population. This could maintain the total amount of K\(^+\) ion efflux, in the neurohypophysial terminals during an action potential, equivalent to that of a naïve rat. Also, the development of tolerance may have an additional component, which maintains normal K\(^+\) ion flow levels by reducing the channel’s response to the systemic alcohol stimulus. So how do these possibilities fit with other chronic effects on the neurohypophysis?

BK channels have been characterized in the rat neurohypophysial terminals and are believed to be involved in the regulation of peptide release (Bondy et al., 1987; Wang et al., 1992). In addition, we have previously shown that chronic ethanol exposure reduces the suppression of vasopressin and oxytocin release from isolated neurohypophysial terminals during an acute ethanol challenge (Fig. 7 and Fig. 9) (Knott et al., 2000). Therefore, if the I\(_A\) current is responsible for the initial repolarization of the membrane, the I\(_A\) current reduction in chronic animals during an acute ethanol challenge may increase the duration of the action potential. In addition, if potentiation of the BK current resulted in the membrane returning to its’ resting potential faster, reducing the potentiation would conversely result in a slower return to the resting potential. Thus,
these alterations in ethanol sensitivity due to chronic exposure would result in a longer depolarization of the membrane, which would enhance the ability of the neuron to release hormone.

Investigators have tried for many years to understand how blood alcohol levels lethal to a naïve system, can be found in individuals admitted to hospitals for treatment of alcoholism. It is our belief that over time, the mammalian system attempts to maintain homeostasis in the body by adapting to the physiological changes which result from chronic exposure to EtOH. These, for lack of a better name, readaptations may play a role in the physiological system’s attempt to correct for an ever present stimulus, such as circulating blood alcohol concentrations which are high enough to normally elicit a response.

Although the results presented in this manuscript may help in identifying how chronic ethanol exposure may be effecting the components that control release, we are not suggesting that they are the only components effected. Other channels such as N- and L-type calcium channels that are also involved in controlling release, and are acutely effected by ethanol challenges must also be investigated. In addition, although we have shown evidence that characteristics of the I_A and BK channels have been changed due to chronic ethanol exposure there are facets of these results that are still left to be investigated. These may include investigation of possible conductance or channel population changes of the BK channel or beta sub-unit substitutions in either BK or I_A, which may alter the sensitivity of the channels toward ethanol. It is our hope that we have begun to provide some initial answers to the question of how molecular alterations could
result in modifications which enable a physiological organism to withstand levels of ethanol known to be lethal to naïve systems.
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Chapter 4

Conclusions
Conclusions

The purpose of this thesis was to study the effects of chronic ethanol exposure on a physiological function that is affected by an acute ethanol challenge and is known to have control of a behavior. The neuropeptide AVP, which is released into the bloodstream via the neurohypophysis fits this criterion. An increase in plasma AVP concentrations results in the retention of water (Eisenhofer and Johnson, 1982) and as previously stated, acute ethanol challenges are known to inhibit the release of AVP from the neurohypophysis (Wang et al., 1991 a,b). Therefore, any inhibition of AVP release would consequently reduce an organism’s water content by inducing urination.

I examined the effect that chronic ethanol exposure has on ethanol’s acute modulatory action at two physiological levels: 1) the release of neuropeptides from the neurohypophysial gland and 2) potassium ion flow through the BK and I_A channels of the neurohypophysis. The main conclusions from this research are that chronic ethanol exposure: 1) reduces the acute ethanol suppression of bath neuropeptides’ release in the intact excised neurohypophysis and isolated neurohypophysial terminals, 2) but does not change baseline release, 3) reduces the potentiation of BK current by acute ethanol challenge, 4) reduces BK current density, and 5) induces acute ethanol sensitivity in I_A channels.
The intent of the research in Chapter Two was to determine if chronic exposure to ethanol would affect a physiological system already known to be modulated by an acute ethanol challenge. The release of AVP and OT from the neurohypophysial system had been shown to be inhibited by an acute ethanol challenge (Wang et al., 1991a,b, Hashimoto et al., 1985). Chronic ethanol exposure resulted in a reduction of the acute ethanol suppression of both AVP and OT release (Fig. 7, Fig. 9, and Fig 10). This indicated that there was a non-specific release mechanism, involved in the control of neuropeptide release that developed tolerance. In addition, artificially raising blood alcohol levels to those observed in the chronic diet animals did not produce the reduced suppression of AVP release during an acute ethanol challenge, observed from animals that were chronically exposed (Fig 8). Also, unpublished results (Fig. 20) show that in an ethanol naïve animal, BK current potentiation with 50 mM ethanol was eliminated during exposure to the drug within 8-10 minutes, with a subsequent ethanol exposure 10 minutes after removal of the drug producing a reduced potentiation (20%) of the current. This is unlike the tolerance seen in Chapter Three. In this chapter, the animal’s exposure to a > 30 mM BAL would be considered the BK channel’s first ethanol exposure. These channels were subsequently rinsed for 30-60 minutes after the neurohypophysial terminals were removed from the animals and dissociated. A subsequent challenge with 50 mM ethanol did not potentiate the BK currents from these chronically exposed animals. The differences in sensitivity during a successive exposure to 50 mM ethanol would suggest that the ethanol tolerance produced during the prolonged acute exposure
meets the acute tolerance criteria proposed by Kalant (1998). Therefore the type of tolerance seen in Chapters Two and Three can not be acute tolerance.

The reduced suppression of neuropeptide release was observed whether the intact neurohypophysis or isolated neurohypophysial terminals were examined. This suggests that although chronic ethanol exposure may produce a myriad variety of changes in the environmental milieu of the intact gland, these changes would not be important to the mechanism(s) controlling release that have developed tolerance to acute ethanol challenges. In summary, the results in Chapter Two indicate that chronic exposure to ethanol induces tolerance to acute exposures: (1) at the level of neurohypophysial peptide release, with the mechanism affected being a general release mechanism, and (2) that the components controlling release, which have developed tolerance, can be isolated to the axon terminal. But what might these components be?

There is evidence suggesting that neurohypophysial peptide release can be affected by a calcium-activated ion channel (Lee et al., 1992). Therefore, if 1) ion channel modulation could induce variation in the amount of hormone being released from the neurohypophysial terminals, and 2) ion channels are the driving force behind the AP (Hodgkins et al., 1949), then the focus of my research seemed to be better served by shifting my attention to the channels in the neurohypophysial terminal known to be involved in regulation of the AP which are acutely effected by ethanol.

In Chapter Three, I focused on the effect of chronic ethanol exposure on the acute potentiation by ethanol of potassium channel currents for three reasons; 1) these channels are known to modulate the duration and frequency of AP spikes (Wong and Prince, 1981)
and after initiation of an AP, induce repolarization of the membrane in neurohypophysial terminals (Thorn et al., 1991; Wang et al., 1992), 2) ethanol has been shown to alter action potentials (Kalant 1975; Oakes and Pozos, 1982), and 3) we had previously shown that chronic ethanol exposure altered a mechanism involved in the release of neurohypophysial hormones (Knott et al., 2000). Therefore chronic exposure to ethanol may modulate the acute effects of ethanol on potassium channel currents and the action potential.

Following chronic ethanol exposure, alcohol sensitivity of the BK channel was reduced. Although the dose dependent response of the neurohypophysial BK channel to acute ethanol challenges previously reported by Dopico et al. (1996b), is evident in both naïve and chronic animals, the alcohol sensitivity threshold for the chronic animals is significantly higher. This effect was not due to a change in either voltage sensitivity or activation kinetics in the chronic group as these characteristics were examined and no significant differences were found between the chronic and naïve groups.

A second effect on the BK channel is a reduction in the current density of the chronically exposed group. Because chronic exposure to ethanol of the L-type calcium channel, known to be inhibited by an acute ethanol challenge in PC12 cells (Grant et al., 1993, Mullikin-Kilpatrick and Tresitman, 1993) and neurohypophysial terminals (Wang et al., 1991a), results in the up-regulation of this channel in PC12 cells (Gerstin et al., 1998), a reduction in BK current density could be related to a reduced channel population. An alternative to a change in channel population could be a reduction in the
BK channel’s conductance. Either of these postulations could explain the reduction in BK current density, but neither is addressed in this thesis.

Chapter Three also looks at the effect of chronic exposure to ethanol on a second potassium channel, $I_A$, reported to be relatively insensitive to acute ethanol challenges. The data showed that chronic exposure induced sensitivity to an acute ethanol challenge of 100 mM or greater. Although in a naïve animal this may be a high concentration of ethanol to use in an acute challenge, BK channels in chronic animals respond to 100 mM ethanol similarly to naïve BK channels challenged with 50 mM ethanol (Fig. 14). Therefore, challenges of chronic animals with an alcohol concentration of 100 mM should be considered physiologically relevant for the chronic group. Other $I_A$ characteristics such as inactivation kinetics and channel density were not affected. In summary, the results in Chapter Three show that following chronic exposure to ethanol the response to acute ethanol challenges in both BK and $I_A$ channels have been modified and that the current density of BK channels has been reduced. But how the chronic ethanol exposure induced modifications in the BK and $I_A$ channel’s response to acute ethanol challenges is an open question.

To begin to solve this question, we must first understand the characteristics that define a channel population’s subtype. A family of calcium-dependent BK channels was first described by the Levitan laboratory (Reinhart et al., 1989). The group was able to identify two types of BK channel, by using rat brain plasma membrane vesicles incorporated into lipid bilayers, which could be characterized by their conductance and toxin sensitivity. In the hypothalamic magnocellular neurons, which project axons from
the supraoptic and paraventricular nuclei to the neurohypophysial terminals and control the release of AVP and OT, investigators have identified two distinctly different BK channels (Type I and Type II) (Wang et al., 1992; Dopico et al., 1999). These channel sub-types have been characterized by their gating kinetics, with the Type I BK channel having faster gating kinetics than the Type II channel (Reinhart et al., 1989; Wang et al., 1992; Dopico et al., 1999). The two BK channel sub-types also differ in their location and sensitivity to both ethanol and charybdotoxin (Dopico et al., 1996a). The Type I BK channel is found in the soma, is insensitive to ethanol (up to 100 mM) and is inhibited by charybdotoxin (Dopico et al., 1999). The Type II BK channel is found in the axon terminal and its sensitivities to ethanol and charybdotoxin are reversed (sensitive to ethanol and insensitive to charybdotoxin) (Wang et al., 1992; Dopico et al., 1996; 1999).

A variety of investigators have shown that BK channel kinetics and charybdotoxin sensitivity can be modified with beta subunit substitutions. Co-expression of the mSlo alpha subunit with the human β1 reduces charybdotoxin sensitivity and induces a slow inactivation of the BK channel, while co-expression with the β3 clone dramatically increases the inactivation kinetics and charybdotoxin sensitivity (Xia et al., 1999). In addition, expressing the hSlo alpha with the human β4 reduces BK activation kinetics (Brenner et al., 2000) and finally, expressing the hSlo alpha with the human β2 reduces the activation kinetics and charybdotoxin sensitivity (Wallner et al., 1999). Therefore, since the kinetics have not been changed in the animals chronically exposed to ethanol it is unlikely that there has been a beta subunit substitution by the betas that have been identified. But this does not rule out the possibility that an as yet unidentified beta subunit
or a splice variant of an existing one is being substituted. In addition, the populations of beta subunits that may or may not exist in the soma and axon endings have not yet been identified. If and when these populations are identified and subsequently cloned, an expression system could be used to examine different combinations of alpha and beta subunits and ascertain whether beta substitutions might be part of the physiological adaptations induced by chronic exposure to ethanol.

An alternative hypothesis is that ethanol may work through a second messenger pathway and that chronic exposure may either modify a component of the pathway or the pathway’s final target on the subunit. Unpublished data from our laboratory during investigations using the BK *bslo* smooth muscle clone in an oocyte expression system has shown us that okadaic acid, a phosphatase 1 and 2A inhibitor, prevented potentiation of the channel current during exposure to an acute 50 mM EtOH challenge. These results support the second messenger theory, but we were not the first group to examine the effects of phosphatases on BK channel activity. In fact, over the past decade, investigators have produced a considerable amount of data suggesting that ethanol’s effect on the BK channel is generated via a phosphorylation pathway (see below). Although there is no agreed upon consensus, scientists are divided into two related but opposing camps; those who favor the idea that ethanol enhances BK channel activity by kinase driven phosphorylation of the alpha subunit and those that favor enhancement by phosphatase driven dephosphorylation.

The research in support of the kinase driven enhancement has been problematic as investigators have identified at least two kinases, PKA and PKC, which induce enhanced
BK channel activity (Reinhart et al., 1991; Levitan, 1994; Jakab et al., 1997). Single channel recordings from cloned pituitary (GH3) tumor cells were used to examine the effects of intracellular signal transduction on ethanol enhancement of the BK channel (Jakab et al., 1997). The investigators showed that PKC inhibitors and AMP-PNP (a nonhydrolyzable ATP-analogue) blocked a 30 mM ethanol induced enhancement of the BK channel. In addition, single channel recordings of BK channels in lipid bilayers have shown that PKA downregulated Type II channels in most cases and upregulated Type I (Reinhart, et al., 1991). However, they showed as well that phosphatase 2A could modulate BK Type I activity without PKA being present, and also restore Type II BK activity. Therefore, although it appears that enhancement of BK channel activity can be induced by kinase activity, there seems to be support for two different possible pathways, a view also endorsed in a review by Levitan (1994). But, even though kinase enhancement of BK channels has its supporters, phosphatase involvement can not be ruled out, as was evidenced in Reinhart’s work (1991).

The idea of phosphatase controlled enhancement of BK channel activity has been gaining strong support with most of the focus aimed at inhibitory G protein activation of phosphatases (Armstrong and White, 1992). Early in the decade, the Armstrong group used GH4C1 pituitary cells to examine peptide enhanced stimulation of guanylyl cyclase activity and membrane conduction of potassium. They showed that peptide stimulation of BK channels could be blocked with cAMP, and that the block could be reversed and the channel potentiated by cGMP. In addition, the cGMP induced potentiation could be blocked with either PKG inhibitors or okadaic acid (White et al, 1991; 1993). Other
investigators, studying smooth muscle BK channel activity, have shown that cGMP-PK needed both ATP and cGMP to increase BK activity (Robertson et al., 1993), that the probability of a BK channel being open can be enhanced with either a cGMP kinase activator or catalytic subunit of phosphatase 2A, but not with a cAMP kinase activator, and that the enhancement can be inhibited with a cGMP kinase inhibitor or okadaic acid (Zhou et al., 1996).

These results suggest that BK channel activity is modulated by the phosphorylation state of the channel. But how does the phosphorylation state of the channel relate to ethanol activity?

The BK channel has serine residues which might be phosphorylated when the channel is in its baseline state. Hence, ethanol may potentiate the current by activating phosphatases to dephosphorylate the BK channel. Therefore, exposure to chronic ethanol might induce modifications in either the alpha subunit, preventing access of the target residues to the phosphatase, or the secondary messenger pathway that activates the phosphatase, thereby reducing it’s ability to dephosphorylate the residues.

The effect of chronic ethanol exposure on the $I_A$ channel is a more difficult phenomenon to address. It has never been shown that drug sensitivity in a previously insensitive channel could be induced by chronic exposure, though this is exactly what has happened in the case of the neurohypophysial terminal’s $I_A$ channel. Explaining this phenomena is difficult because of two known characteristics of $I_A$ channels chronically exposed to ethanol; 1) chronic ethanol exposure does not modify inactivation kinetics
(Fig. 18a) or 2) alter current density (Fig. 18b). But these results do not rule out the channel being modified by an associated beta subunit as an alternative.

As previously mentioned, BK channel characteristics can be modified by associated beta subunits. But the BK alpha subunits are believed to interact with the beta subunits through the alpha’s N-terminus S0 hydrophobic region (Meera et al., 1997), a region that the IA channel is not reported to have. Although there is no conclusive evidence that IA channels in the neurohypophysial terminals have associated beta subunits, the possibility should not be rejected. There is a family of voltage-gated K⁺ channel beta subunits generated by alternative splicing, that are expressed from three genes (Leicher et al., 1998), and are believed to be cytosolic (Trimmer, 1998). Some of these beta subunits have N-terminal sequences similar to that found in the rapidly inactivating Shaker (A-type) channels (England et al., 1995; Morales et al., 1995; Leicher et al., 1996). The structure is known as the “ball” domain, which upon depolarization inactivates the channel by occluding the channel pore (Zagotta et al., 1990; Isacoff et al., 1991). Recently, it has been shown that coexpression of KV beta 1.1, 2, and 3 with various KV alphas can induce or accelerate inactivation in a variety of KV delayed rectifier and IA channels (Heinemann et al., 1996; Leicher et al., 1998). Also, in hippocampal and striatal neurons from knock-out mice, inactive KV beta 1.1 subunits lead to reduced IA channel activity (Pongs et al., 1999). Therefore, since KV beta subunits can alter alpha characteristics and are known to associate with specific cytoplasmic sites of KV alpha subunits (Yu et al., 1996; Sewing et al., 1996), a beta substitution during chronic exposure to ethanol may produce the decrease in neurohypophysial IA channel
current observed during the acute ethanol challenge in Chapter three. In addition, although there isn’t any evidence of a splice variant in the I\textsubscript{A} neurohypophysial channel population, a hydrophobic point mutation in an ethanol-insensitive voltage-gated potassium channel (K\textsubscript{v}3.4) was able to induce a dose-dependent ethanol sensitivity (inhibition), similar to that of the Shaw2 channel (Covarrubias et al., 1995). Therefore, although I reiterate that it has not been reported, chronic exposure may be able to induce translation of an I\textsubscript{A} alpha splice variant that is sensitive to ethanol. Nevertheless, the evidence is indisputable, chronic exposure to ethanol induces acute sensitivity to ethanol (inhibition) in the neurohypophysial I\textsubscript{A} channel.

One additional issue is the effect of chronic ethanol exposure on the lipid bilayer. It has been reported that chronic exposure to ethanol increases the cholesterol content in the cytofacial bilayer leaflet of mice administered ethanol for 3 weeks (Wood et al., 1989). Also, increasing cholesterol in reconstituted lipid bilayers reduces the mean open time of the BK channel (Chang et al., 1995; Crowley et al., 2000), reduces conductance by 7% (Chang et al., 1995), and reduces the potentiation of the channel by ethanol (Crowley et al., 2000). These findings suggest that a modification of the lipid environment following chronic exposure may alter the lipid protein interface. These modifications may have a role in the reduced ethanol effect seen in chapters 2 and 3.

So how does the effect of chronic ethanol exposure on the potassium channels of the neurohypophysis, relate to the reduced suppression of neuropeptide release during an acute ethanol challenge?
It has been established that modulation of $I_A$ and BK channels can affect the AP. In the late 1980's research studying the effect of potassium channel blockers showed that both 4AP which specifically inhibits the fast inactivating $I_A$ channel and TEA, a general potassium channel blocker, increase the release of AVP and OT from the intact neurohypophysis (Bondy et al., 1987). Also, tetrandrine which is a BK specific channel blocker slowed membrane repolarization in isolated neurohypophysial terminals (Dopico et al., 1999; Wang and Lemos, submitted), and both TEA and 4AP have recently been shown to increase the breadth of an AP, ~50%, and 1000%, respectively (Wang and Lemos, submitted). Finally, ethanol itself has been shown to alter the repolarization phase of the action potential (Oakes and Pozos, 1982). Therefore, modulation of $K^+$ channel activity by acute and chronic ethanol exposure should have a direct effect on AVP and OT release.

An acute ethanol challenge, at physiological relevant concentrations, on isolated neurohypophysial terminals from naïve animals would have no effect on the $I_A$ channel but would potentiate the BK channel. Thus the membrane would return to its resting potential faster than normal, resulting in a faster repolarization and a narrower AP, subsequently decreasing calcium influx and thus reducing the release of neuropeptide. In the naïve animal this effect could be seen at concentrations up to 100 mM (Figure 19). But, in animals chronically exposed to ethanol the effects of an acute ethanol challenge are more subtle. At a concentration of 50 mM there is no effect on the $I_A$ current and little potentiation of the BK current. This would suggest that there would be minimal effects on the release of neuropeptide, and as can be seen in Figs 7 and 9, an acute challenge
Figure 19. A model of the neurohypophysial terminal action potential during a 100 mM EtOH challenge. A) In the ethanol-naïve animal, potentiation of BK current thins AP resulting in shorter opening of calcium channels. B) In the chronic-ethanol diet animal, reduced potentiation of BK current and slight inhibition of $I_A$ current results in no change in the AP or calcium channel current. Solid line-control condition, dash line-ethanol challenge. Black – action potential, blue – fast inactivating $I_A$ channel, red – calcium-activated BK channel, green – calcium channels. Action potential is modeled after Wang and Lemos, submitted.
with 75 mM has no effect on the release of either AVP or OT. When the ethanol concentration is increased to 100 mM, where it begins to affect the $I_A$ current, there would be a 10-15% inhibition of $I_A$ current combined with a 35-40% potentiation of BK current. The acute ethanol effect on the chronic ethanol animal’s K$^+$ channels may end up offsetting each other (Figure 19), resulting in little or no suppression of release. Although concentrations over 75 mM were not used in our release studies, the electrophysiology data does suggest that higher concentrations of ethanol could produce opposing effects on the AP. However, even blocking the $I_A$ channel with 7 mM 4-AP resulted in only a 20-25% increase in AVP release (Dayanithi and Lemos, personal communication). Therefore, it is likely that an inhibition of release, via the potentiation of BK current, could begin to supercede the $I_A$ inhibition effect (increasing release) during an acute challenge of a chronic animal with 100 or 150 mM ethanol and significantly reduce the amount of hormone released.

In this thesis I am suggesting that chronic ethanol induced modifications, of BK and $I_A$ channel ethanol sensitivity, have a direct relationship on the effect of acute ethanol suppression of neurohypophysial hormone release via their AP modulatory abilities. But, because the release experiments were performed by depolarizing with high potassium and not by electrical stimulation, the suggestion is theoretical. To confirm whether or not the modifications in these channels do in fact, directly relate to the change in suppression, release experiments would have to be carried out by electrical stimulation of the intact excised neurohypophysial gland under all the experimental conditions in chapter 2.
This thesis concentrates only on the effect chronic exposure has on the potassium channels on the neurohypophysis, but these might not be the only channels involved in the control of hormone release that are affected. Two types of voltage-gated calcium channels, L and N, are known to be inhibited by acute ethanol challenges in a dose dependent manner (Wang et al., 1991a,b). In addition, investigators studying PC 12 cells have looked at the effects of chronic ethanol exposure on calcium channels. These investigators established that chronic exposure increased calcium current (Grant et al., 1993) and up-regulated the L-type channel population (Gerstin et al., 1998). Therefore, chronic ethanol exposure may induce modifications in other calcium channel characteristics such as kinetics or ethanol sensitivity, and changes in either of these characteristics would have direct ramifications on the hormone release capabilities of the neurohypophysis. Thus, although I feel that the potassium channel modifications following chronic ethanol exposure have an important modulatory effect on the release of AVP and OT from the neurohypophysis, I think it is unlikely that they are the only components involved in release that have been affected by chronic exposure.

Future research aims should begin to examine the effects of chronic ethanol exposure on; 1) the aforementioned electrophysiological characteristics of calcium channels, 2) BK conductance using single-channel recordings, and 3) the BK channel population. In addition, BK beta subunit populations in naïve and animals chronically exposed to ethanol should be identified and compared. Also, it is possible that a splice variant, similar to the point mutation variant K_v3.4 channel, which induced inhibitory sensitivity to ethanol similar to the Shaw2 (Covarrubias et al., 1995), may be responsible
for conferring ethanol sensitivity on the $I_A$ channel. Therefore, the $I_A$ channel peptide sequences in naïve and chronic animals should be examined and compared for possible splice variants.

Also, the other types of ethanol tolerance, acute and rapid, suggested by Kalant (1998) and in appendix A figure 20, should be examined more thoroughly for us to better understand all the molecular underpinnings of the tolerance phenomenon. Some forms of tolerance may have related, as well as distinctly different, components. Acute tolerance may be the result of desensitization to the effects of ethanol, via repeated interaction with a hypothetical binding site on the channel or a change in the channel’s phosphorylation state, which must occurs within minutes during prolonged exposure. But because Bitran and Kalant (1991) showed that acute tolerance can also manifest during the development of rapid tolerance it is likely that there are either additional components involved or more extensive modifications in the ones affected during the development of acute tolerance. Rapid tolerance which dissipates after 24 hours might entail a change in the kinase or phosphatase populations following a second exposure to the drug, which may alter the baseline phosphorylation state of the channel. It may also involve a change in the cholesterol lipid content of the membrane which has been shown in our laboratory to reduce the potentiation of the BK channel by ethanol (Crowley et al, 2000). All of these possibilities should be examined to further enhance our understanding of the mechanisms that underlie the development of tolerance.

Finally, although I have offered a variety of possible future research aims, most are dependent upon the existence of previously established acute ethanol sensitivity. But,
after seeing the effects of chronic ethanol exposure on $I_A$ channel activity, we must keep ourselves open to the possibility that many physiological components and mechanisms which have never before exhibited ethanol sensitivity, might do so following chronic exposure. Therefore, because acute ethanol sensitivity can be induced through chronic exposure, investigators may have to redefine the criteria used to decide which mechanisms should be examined, when studying the physiological effects of chronic ethanol exposure.
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Appendix A

Long-term acute challenge with ethanol can induce tolerance.

This thesis has focused on the development of tolerance due to chronic ethanol exposure. However, in a review of the current state of the tolerance phenomena, Harold Kalant (1998) proposed a three-tiered hierarchy of acquired drug tolerance; acute, rapid, and chronic. In an early associated experiment, I briefly examined the effect of a long-term acute challenge of ethanol on the BK channel. The results indicated that tolerance can develop during an acute exposure.

Due to the trauma resulting from rupture of the membrane for whole-cell electrophysiology, isolated terminals do not survive for more than 20-25 minutes. Thus, experiments of a longer duration can not be done using the whole-cell technique. Therefore, to examine the acute tolerance phenomena, I used the perforated patch technique which is minimally invasive. In this technique, instead of rupturing the membrane to allow continuity with the cytosol, an anti-fungal (amphotericin B) that creates holes in membranes containing cholesterol is added to the pipette solution. These holes are small enough so that only mono-valent ions are able to pass through, keeping the cytosol and its components intact.

I exposed the terminal to a 50 mM ethanol concentration by pipetting a 1M ethanol solution into the standing bath. To prevent the disruption of the seal when
studying the isolated terminals, ethanol was added to the bath solution at the maximum distance from the terminal. This causes the least turbulence in the bath and optimizes the survival of the preparation. But, because the ethanol is added at a distance from the terminal, potentiation of the BK channel activity is delayed (Fig. 20a,b). Within 2-3 minutes after addition of ethanol the channel showed an initial potentiation of ~80%, which is twice as large as that seen in experiments, where ethanol is applied directly to the terminal with a sewer pipe. This increased potentiation may be due to a bolus effect, in which a higher than expected concentration of ethanol is reaching the terminal prior to total diffusion. Yet, within 3 minutes the potentiation is reduced to ~35-40%, which is similar to that seen in experiments using the pipe perfusion method. The current potentiation then disappeared within an additional 4-5 minutes. Following the loss of ethanol potentiation, a second bolus of ethanol was added to control for possible drug evaporation. Current traces were examined for 20 minutes, and no potentiation of the BK channel was observed. This result indicates that a form of acute tolerance has developed. The terminal was then rinsed for 5 minutes and ethanol added again. The BK current was potentiated ~20%, which is lower than normal, but the potentiation again disappeared within 7-8 minutes, similar to the time frame of potentiation by the first ethanol exposure.

This experiment suggests that an acute form of ethanol tolerance has developed, which can be rinsed out, and that it occurs during the continual application of the drug. What sets this type of tolerance apart from that seen in the chronically exposed animals is that after removal of the drug, a second ethanol exposure results in a reduced response to the drug. This type of tolerance is different because in the chronic animals, the first
Figure 20. Acute tolerance develops in isolated neurohypophysial terminals. HP is –80 mV and stepped to +40 mV with perforated patch recordings of BK current taken every 16 seconds. A) Percent increase in current per minute over the duration of the experiment with three 50 mM acute ethanol challenges. B) BK current amplitude following the first challenge with 50 mM ethanol.
ethanol exposure would be the 30-35 mM range BAL (Table 1) the terminals are continually exposed to. The neurohypophysis which contains these terminals is removed and homogenized in sucrose. The terminals are then placed in a polystyrene coated dish and rinsed in solutions containing no ethanol for at least 30-45 minutes before the electrophysiology is performed. In these BK channels, there is no potentiation of the current during a 50 mM ethanol challenge, unlike the 20% potentiation of BK channel current seen after a 5 minute rinse during the acute tolerance experiment. This suggests that the drug tolerance that develops during a prolonged acute exposure is not the same type as that which develops during long-term exposure, but it does not rule out the possibility that there is a pathway common to the development of both types of tolerance.
Appendix B

Withdrawal reverses chronic EtOH modifications of BK channel EtOH sensitivity

This thesis has shown that following long-term ethanol exposure, acute EtOH sensitivity and current density has been reduced in neurohypophysial BK channels and has suggested that these modifications may have induced the change in AVP and OT release patterns observed during an acute EtOH challenge. But if ETOH is withdrawn from the animals will the BK channel modifications be eliminated and channel density and sensitivity returned to normal?

Many investigators have studied withdrawal at different points in time and examined the physiological response of their interest. Early in the past decade, an investigation team measured the plasma AVP levels of rats within a 72 hour period following the initiation of withdrawal (Hoffman and Dave, 1991). After 8 days of exposure to an ethanol vapor the rats had BAL which ranged from 22 to 39 mM. They found that the plasma vasopressin levels were significantly higher than control levels for 48 to 72 hours, before returning to control levels. This suggests that modifications in the control of AVP release could return to normal following withdrawal.

We investigated the withdrawal phenomenon 14-21 days after the rats have been returned to an ethanol-free diet. At this point the potentiation of BK current in terminals, produced by an acute ethanol challenge, returned to values observed in ethanol-naïve rats (Fig. 21a). In addition, the decreased BK current density observed in terminals from the
Figure 21. BK channel characteristics from animals having gone through withdrawal. (a) Acute challenge with 50 mM EtOH. Data for naïve and chronic animals reprinted from figure 14, withdrawn animals (n=3). (b) Current density comparison. Data for naïve and chronic animals reprinted from figure 17, withdrawn (n=3). Currents measured and isolated as in protocol from figure 11. Capacitance measured as in figures 17 and 18. * p < 0.05
chronically-exposed rats shows a *partial reversal* in rats which have been withdrawn from the drug, within the time frame monitored (Fig.20b).

BK channel sensitivity has returned to pre-exposure levels within 14 days of drug withdrawal, and current density appears to be showing a similar trend, although not yet statistically significant at this time point or 7 days later. The fact that the two parameters recover at independent rates suggests that they are controlled by distinct mechanisms, and this is further supported by the I-A results reported earlier, which showed that changes in sensitivity could occur independent of changes in current density.