Chronic alcohol exposure increases ganglia endogenous morphine levels

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Introduction: We have previously demonstrated that alcohol has the ability to release low levels of endogenously expressed, chemically authentic, morphine from neural tissues.

Material and methods: Presently, we demonstrate that chronic exposure of Mytilus edulis pedal ganglia tissues maintained in organotypic culture to very concentrations of 1 mM and 10 mM ethanol induces a time dependent increase in both endogenous morphine and dopamine (DA) levels.

Results: Chronic incubation of M. edulis pedal ganglia with 3 concentrations of DA resulted in statistically significant elevations of cellular morphine levels, thereby confirming previous studies from our laboratory establishing DA as an essential precursor in the morphine biosynthetic pathway.

Conclusions: By understanding multiple debilitating effects of alcohol on “morphinergic” signaling, we may understand the ravages of neural processes associated with alcohol abuse and how its treatment may be made more effective.

Key words: endogenous morphine, dopamine, alcohol, ethanol, ganglia.

Introduction

The positive reinforcing effects of alcohol consumption are mediated through complex interactions between multiple CNS systems [1-11]. These systems appear to be functionally entrained within the A10 mesocortical-mesolimbic DA-ergic cell group. A10 DA neurons have also been implicated in the development of dependencies to relatively broad spectrum of addictive compounds including opiates, psychostimulants, and nicotine [12]. The neurochemical effects of alcohol on A10 neurons appear to be partially mediated via release of candidate neuromodulators/ neurotransmitters including endogenous opioid peptides, S-HT, glutamate, GABA, and acetylcholine [2, 13]. Mechanistically, these same neurochemical systems represent valid therapeutic targets to curb alcohol cravings and maintain abstinence in chronic abusers [14-17].

In particular, there appear to multiple levels of functional crosstalk between alcohol, opioid receptors, and A10 DA neurons [18, 19]. For example, the reinforcing behavioural properties of mu and delta opioid receptor selective agonists have been functionally mapped to specific regions of the nucleus accumbens (NAC) and prefrontal cortex [20, 21]. Several lines of evidence indicate alcohol-mediated disruption of normal

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opiod signaling mechanisms within A10 neurons. These include studies demonstrating direct effects of alcohol on the binding properties of opioid receptors, modulation of opioid peptide synthesis and secretion, and the formation of alcohol-derived isoquinoline condensation products [22]. The widespread clinical usage of the mu opioid receptor antagonist naltrexone for alcohol abuse provides strong confirmation of intimate functional relationships of alcohol, opiates, and A10 DA neurons [14].

In light of the discussion presented above, we have previously demonstrated that alcohol has the ability to release low levels of endogenously expressed, chemically authentic, morphine from various tissues, including those of human origin [23, 24]. The potential importance of these observations may lie in their suggestive implications for the regulatory importance of cellular homeostasis maintained by ongoing “morphinergic” signaling events [25-27]. The critical importance of DA as an essential precursor in the morphine biosynthetic pathway lends further credence to these contentions [25-27]. More recent studies from our laboratory demonstrate that other substances of abuse (cocaine and nicotine) also appear to have the ability to release endogenous morphine from cells and at the same time promote compensatory synthesis of the alkaloid [23, 24, 28, 29].

In sum, previous work from our laboratory has demonstrated that multiple substances of abuse have the ability to enhance endogenous morphine processes at a common step, e.g., release from cellular stores, in invertebrate and human tissues [23, 24, 28, 30]. In the present report we further demonstrate that chronic exposure of neural tissues to ethanol induces a time dependent increase in both endogenous morphine and DA levels. By understanding multiple debilitating effects of alcohol on “morphinergic” signaling, we may understand the ravages of neural processes associated with alcohol abuse and how its treatment may be made more effective.

Material and methods

Tissue preparation and pharmacological treatments

*Mylitis edulis* were collected from the local waters of Long Island Sound and maintained as previously described in detail [31]. Pooled pedal ganglia from 20 animals were separately incubated with 1 mM or 10 mM ethanol, or vehicle for 1 h, 2 h, 6 h, 12 h, and 24 h. Ethanol and vehicle treated ganglia were harvested, washed, and extracted as described below. In an additional study, pooled pedal ganglia from 20 animals were separately incubated with DA at 10^{-2}M, 10^{-4}M, and 10^{-6}M for 18 h. DA treated ganglia were harvested, washed, and extracted as described below.

**Extraction of treated pedal ganglia**

For morphine and DA analysis, pooled pedal ganglia samples were dissolved in 1 N HCl and homogenized by sonication. Tissue homogenates were extracted with 5 ml chloroform/isopropanol 9 : 1 for 5 min at 30°. Following centrifugation at 3000 rpm for 15 min, the upper aqueous phase was reserved for morphine and DA quantification and vacuum dried using a Centrivap Console (Labconco, Kansas City, Missouri). Dried extracts were dissolved in 1 ml 0.05% trifluoroacetic acid (TFA), clarified, and passed through activated Waters Sep-Pak Plus C-18 cartridges. Elution was achieved utilizing an H2O/CH3CN/TFA solution (89.5% : 10% : 0.05%, v/v/v). Eluted fractions were dried prior to morphine and DA analyses.

**RIA quantification of extracted morphine**

For analysis of morphine in extracted and dried fractions, we employed a commercially available RIA kit obtained from Diagnostic Products Corporation. The morphine RIA is a solid phase competitive binding analysis, whereby 125I-labeled morphine competes for immobilized antibody binding sites with extracted morphine in the test sample (USA) [32-36]. The detection limit is 0.5 ng/ml.

**Combined HPLC and electrochemical detection (ECD) of dopamine**

The combined HPLC-ECD analyses for DA quantification were performed with a Waters 626 pump (Waters, Milford, MA) and a C-18 Unijet microbore column (BAS). A flow splitter (BAS) was used to provide the low volumetric flow-rates required for the microbore column. The split ratio was 1/9. Operating the pump at 0.5 ml/min, yielded a microbore column flow-rate of 50 µl/min. The injection volume was 5 µl. Dopamine quantification was achieved via using amperometric detector LC-4C (BAS, West Lafayette, Indiana). The microbore column was coupled directly to the detector cell to minimize the dead volume. The electrochemical detection system used a glassy carbon-working electrode (3 mm) and a 0.02 Hz filter (500 mV; range 10 nA). The cell volume was reduced by a 16-µm gasket. The chromatographic system was controlled by Waters Millennium32 Chromatography Manager V3.2 software and the chromatograms were integrated with Chromatograph software (Waters).

**Statistical analysis**

A two-way ANOVA was used for statistical analysis of treated pedal ganglia tissues. Each
experiment was performed 4 times. The mean value was combined with the mean value taken from 4 other replicates. The SEM represents the variation of the mean of the means.

Results

Chronic incubation of *M. edulis* pedal ganglia maintained in organotypic culture with very low concentrations of 1 mM or 10 mM ethanol for 6 h, 12 h, and 24 h resulted in statistically significant elevations of cellular morphine levels (1.74 ±0.73 ng/mg, 5.26 ±0.68 ng/mg, and 7.39 ±0.94 ng/mg were observed at 6 h, 12 h, and 24 h, respectively) (Figure 1). In effect, we observed apparent 200-400% increases in cellular morphine concentrations after 12 h and 24 h incubation with 1 mM or 10 mM ethanol. These observations indicate that chronic incubation with very low concentrations of ethanol promote cellular biosynthetic mechanisms to increase endogenous morphine.

Chronic exposure of invertebrate neural ganglia to the same low concentrations of ethanol increased DA levels at the 24 h time point as well (Figure 2). As depicted in Figure 2, incubation of *M. edulis* pedal ganglia with 1 mM or 10 mM ethanol equivalent to 0.003% v/v and 0.03% v/v, respectively, for 6 h, 12 h, and 24 h resulted in a significant elevation of cellular DA levels at the 24 h time point (19.33 ±6.54 ng/mg, 38.75 ±7.36 ng/mg for 1 mM ethanol at 6 h, 12 h, and 24 h, respectively). We observed an apparent 100% increase in cellular DA concentrations after 24 hr incubation with 1 mM ethanol vs. control (17.53 ±6.4 ng/mg vs. 38.75 ±7.36 ng/mg, respectively). The 24 h increase in cellular DA concentration is consistent with the role of DA as an essential precursor in the morphine biosynthetic pathway [27].

Finally, chronic incubation of *M. edulis* pedal ganglia maintained in organotypic culture with 3 concentrations of DA at 10–7M, 10–6M, and 10–5M for 18 h, resulted in statistically significant elevations of cellular morphine levels: 4.2 ±1.7 ng/mg, 7.6 ±1.9 ng/mg, 9.3 ±2.5 ng/mg, respectively (Figure 3). These observations confirm previous studies from our laboratory establishing DA as an essential precursor in the morphine biosynthetic pathway.

Discussion

In the present report we demonstrate that chronic exposure of *M. edulis* neural tissues maintained in organotypic culture to very low concentration of 1 mM ethanol equivalent to 0.003% v/v ethanol induces a time dependent
increase in both endogenous morphine and DA levels. Interestingly, acute 1 hr exposure to the same concentration of ethanol produces a marked reduction of endogenous morphine levels by < 50% vs. controls and is consistent with the ability of 1 mM ethanol to promote evoked release of morphine into the cell culture medium, including those involving human white blood cells [23, 24]. Furthermore, these experiments suggest that a portion of neural DA stores may be targeted for morphine biosynthesis and are open to physiological modulation, depending on the particular type of stimulus.

Previous studies have shown that CYP2D6 is one of the key enzymes for morphine biosynthesis in animals, including human [26, 37]. In support of our current observations, others have shown that in post mortem analyses, brain tissues of chronic alcohol abusers express high levels of CYP2D6 [38]. Thus, a functional linkage between chronic alcohol exposure in vivo and endogenous morphine expression may be mediated via the increased expression of this key morphine biosynthetic enzyme [37].

Our overall testable hypothesis reciprocally linking endogenous morphine production and release to cellular processes necessary for homeostasis of DA-ergic signaling is supported by a gene expression/microarray study of human genes in primary cultures of human leukocytes exposed to pharmacological concentrations of morphine (10^{-6} M) [39]. Morphine effected differential changes in mRNA levels of candidate enzymes involved in DA/catecholamine and/or endogenous morphine synthesis. We observed an approximate 50% reduction in tyrosine hydroxylase, COMT, and CYP2D6 mRNA levels, as compared to control values. In contrast, levels of L-DOPA decarboxylase and DA β-hydroxylase (DDC and DBH, respectively) mRNAs were relatively unchanged vs. control values, indicating a selective pharmacological effect of morphine on key enzyme activities involved in both endogenous morphine and DA expression in PMNs. Secondary validation of morphine-mediated changes in the gene expression/microarray analysis was provided by real-time Taqman RT-PCR of CYP2D6 mRNA expression in primary cultures of human PMNs treated with morphine. The RT-PCR analysis yielded equivalent reductions in cellular levels of CYP2D6 mRNA of approximately 50%, thereby confirming the inhibitory effect of morphine on CYP2D6 mRNA expression monitored by gene expression/microarray analysis. Furthermore, the study also demonstrated that the morphine action was mediated by NO, providing additional support for the functional linkage of endogenous morphine, the μ_{3}, and cNOS activation.

In view of the reinforcing properties of alcohol in combination with the in vitro observations from our present study, the effects of alcohol on endogenous morphine and DA expression appear to be both time and dose dependent. Reciprocally, preclinical animal studies demonstrate dose-dependent effects of administered morphine on the rate alcohol self administration [40, 41]. Finally, preclinical studies have led to the adoption of opioid antagonist therapies employing naltrexone for maintenance of alcohol abstinence in humans [42].

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