Supplemental Data Figure 2

Ethanol reversal of cellular tolerance to morphine in rat locus coeruleus neurons

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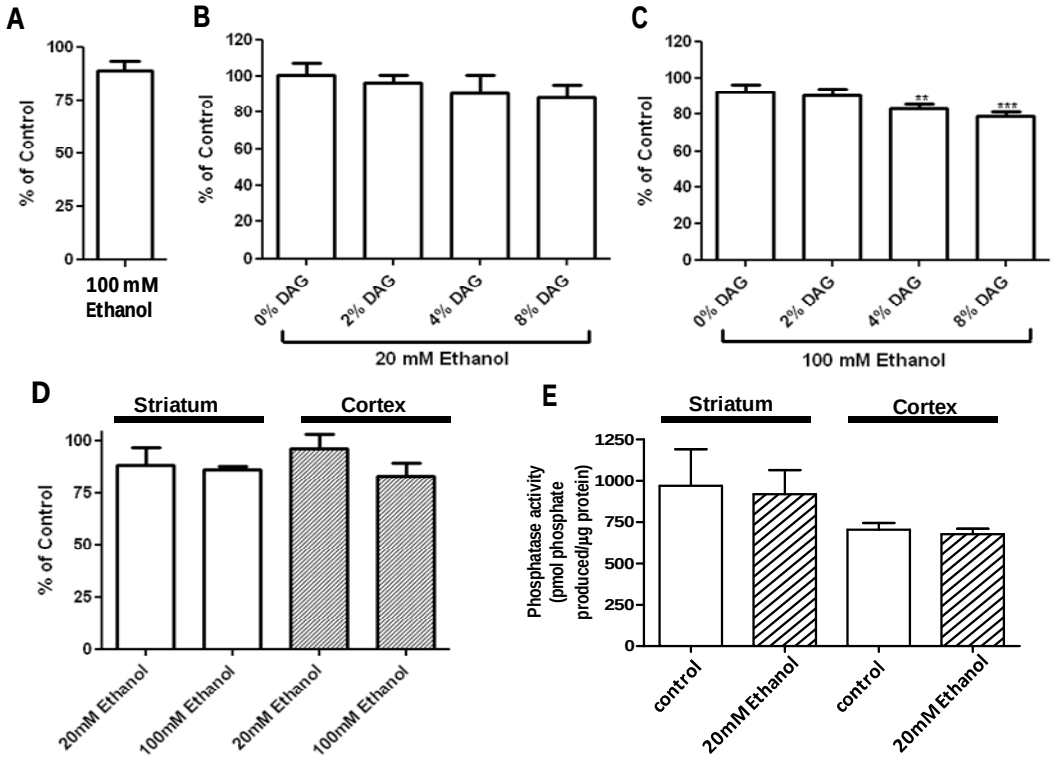
Molecular Pharmacology



Lack of effect of ethanol on purified PKCα activity and mouse brain phosphatase activity

A-D PKC activity A nonradioactive PKC detection kit (PepTag assay kit; Promega) was used to measure kinase activity *in vitro* according to the manufacturer's instructions. Recombinant PKCα protein (10 ng) in lipid vesicles containing diacylglycerol (DAG; 0%, 2%, 4% or 8%), phosphatidylcholine (80%, 78%, 76% or 72%) and phosphatidylserine (20%) were prepared and enzyme activity determined in the presence and absence of ethanol by incubation with Peptag C1 substrate peptide for 30 min at 30°C. Phosphorylated peptide was resolved by SDS-PAGE and quantified by

densitometry after detection by UV illumination. **A** Ethanol (100 mM) did not alter PKCα activity in the absence of lipid vesicles. **B** Ethanol (20 mM) had no significant effect on PKCα activity in the presence of lipid vesicles. **C** Ethanol (100 mM) caused a decrease in PKCα activity which did reach significance however it cannot be concluded that ethanol reverses cellular tolerance by inhibiting PKCα activity. Due to the highly intoxicating effects of ethanol (100 mM), the effect on PKCα activity maybe unrelated to the reversal of tolerance. **D** Ethanol (20 or 100 mM) did not inhibit endogenous PKC activity in supernatants from mouse cortex and striatum. mouse brains were homogenized using a Polytron® homogenizer, in PKC extraction buffer (25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). The homogenate was centrifuged at 14,000 x g at 4^{o}C for 5 min and the supernatant saved. Supernatant was passed over a 1 ml column of DEAE cellulose, pre-equilibriated in PKC extraction buffer and washed through with 2 ml PKC extraction buffer. The PKC containing fraction was eluted using PKC extraction buffer containing NaCl (200 mM). PKC activity was analysed as above. Ethanol (20 and 100 mM) had no significant effect on endogenous PKC activity in the cortex or the striatum. **E** Ethanol (20 mM) did not alter phosphatase activity in supernatants from mouse cortex and striatum. Phosphatase activity measured with a kit-based colorimetric assay (Promega Serine/Threonine Phosphatase Assay System), according to the supplier's instructions. Brain tissue from mouse striatum or cortex was dissected and frozen at -80°C. For the assay, tissue was rapidly thawed and homogenized using a Polytron® homogenizer, and the resulting homogenate centrifuged at 100,000 x g at 4°C for 1h. The supernatant was subsequently used for the phosphatase assay, which measures the production of free phosphate from a phosphopeptide substrate as the development of green colour with a molybdate dye, and is determined using a plate reader. Ethanol was added to the brain supernatants just before the plate assay was begun.