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AGE-DEPENDENT MODULATION OF GLUTAMATERGIC SYNAPTIC TRANSMISSION BY ETHANOL IN THE PREFRONTAL CORTEX

Degree Project in Medicine

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Gothenburg, Sweden 2017

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Table of Contents

List of abbreviations

ACh – Acetylcholine
AChR – Acetylcholine receptor
aCSF – Artificial cerebrospinal fluid
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APA – American psychiatric association
AUD – Alcohol use disorder
BF – Basal forebrain
BNST – Bed nucleus of stria terminalis
BRS – Brain reward system
CeA – Central Amygdala
DSM 5 – Diagnostic and Statistical Manual of Mental Disorders 5th edition
EPSC – Excitatory postsynaptic current
EPSP – Excitatory postsynaptic potential
GABA – γ-amino-butyric acid
$GABA_AR$ – γ-amino-butyric acid A receptor
GlyR – Glycine receptor
$5\text{-}HT_3R$ – Serotonin receptor
MEC - Mecamylamine
nAc – Nucleus accumbens
nAChR – Nicotinic acetylcholine receptor
NBQX - 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA – N-methyl-D-aspartate
NMDAR - N-methyl-D-aspartate receptor
PFC – Prefrontal cortex
PND – Postnatal day
PS – Population spike
SEM – Standard error of the mean
SN – Substantia nigra
VP – Ventral pallidum
VTA – Ventral tegmental area

ABSTRACT

AGE-DEPENDENT MODULATION OF GLUTAMATERGIC SYNAPTIC TRANSMISSION BY ETHANOL IN THE PREFRONTAL CORTEX

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Introduction: Dysregulation of the prefrontal cortex (PFC) is a crucial feature of addiction. Alcohol and nicotine are commonly co-abused and evidence suggests that they interact on the nicotinic acetylcholine receptor (nAChR). Impaired prefrontal function in mice, due to chronic ethanol (EtOH) exposure, have been linked to changes in glutamatergic neurotransmission in the PFC. **Aim:** The aim of this project was therefore to analyse, at the synaptic level, if differences exist in the PFC of young rats compared with adult regarding glutamatergic neurotransmission – under normal conditions and following treatment with nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine (MEC), and/or EtOH. **Methods/results:** Using electrophysiological recordings in brain slice preparations we analyzed the effects of EtOH treatment on population spike (PS) amplitude in the juvenile rat PFC and found that treatment with 50 mM and 100 mM but not 20 mM EtOH significantly depressed PS amplitude. We also analyzed the effect of treatment with MEC (10 μM), which by

itself had no effect on PS amplitude, while the EtOH-induced depression was blocked in slices treated with MEC. Finally, we analyzed the differences between juvenile and adult rat slice preparations in treatment with 50 mM EtOH. There was no significant depression in the adult slice preparations. **Conclusions**: EtOH depressed PS amplitude in the PFC of juvenile rat slice preparations. Treatment with MEC blocked this depression strongly indicating that it was dependent upon nAChRs, presumably on GABAergic neurons. EtOH induced a significant depression of PFC output in juvenile, but not adult rat slice preparations. Data from the present study further supports the notion that the adolescent brain is more sensitive to the effects of EtOH. Thus underscoring the importance of developing effective preventive interventions in order to postpone alcohol-debut as late as possible.

Key words: Addiction, Population spike, Adolescence, Electrophysiology

INTRODUCTION

Preface

Alcohol is, and has traditionally been a part of most societies and cultures. Chemical analysis of ancient pottery from China have revealed evidence that fermented beverages were being produced at least as early as 7000 years before Christ (1).

Alcohol is the third leading risk factor for disease and mortality in Europe, the first being tobacco, the second high blood pressure (2). Alcohol can cause (among other diseases and health issues) liver disease, cardiovascular disease, cancers and all types of injury (intentional and unintentional) and harms not only the consumer, but also family, colleagues and the community in terms of costs providing health care etcetera.

In 2004 the global average consumption for adults was 6.1 liters of pure alcohol per capita (in Sweden, one standard drink unit contains 15 ml (12 g) of pure alcohol (3)). In the European union, the average consumption for adults is 12.5 liters of pure alcohol per capita – the highest alcohol consumption in the world (2). In Sweden, the direct costs alone (e.g. health-care, preventive work, emergency services, social services and other) of the consumption of alcohol and drugs, were calculated to almost 30 billion SEK in 2003 (4).

Indeed, alcohol abuse cause major health problems and the costs for society are huge. It is therefore of great importance to find new, more effective, strategies of treatment

and prevention. Reduced consumption of alcohol and tobacco is included in one of the eleven public health initiatives that the Swedish parliament in 2003 enacted (5).

Extensive studies have been performed, declaring a close association between nicotine and alcohol. Nicotine and alcohol are commonly co-abused and early onset smoking is strongly correlated with alcohol abuse and dependence later in life (6). Adolescents initianting either smoking or alcohol consumption is at a significant higher risk of initiating the other shortly thereafter (7).

The brain undergoes important structural changes during adolescence. The grey matter volume and thickness peak around ages 12-14, and than declines as a result of selective synaptic pruning. The white matter volume increases during adolescence, especially in the fronto-parietal regions, reflecting growth and enhancement of neural pathways (8). The maturation of neural circuitry in the prefrontal cortex (PFC), is linked to advancements in cognitive functions (e.g. working memory, planning, inhibitory control and problem solving) and behavior (8). However, adolescence is also a period related to increased risk-taking and an elevated activity in emotion processing centers, suggesting that emotional context is more likely to influence the adolescent compared to adults (8, 9). These traits may be related to an increased sensitivity to rewarding outcomes and therefore an increased vulnerability towards drug-use and alcohol consumption. One theory, supported by fMRI studies (10), is that hyperactivity in the striatum (due to an increased dopamine-release) during adolescence may cause them to seek further reward, resulting in a reinforcing cycle of drug seeking. Furthermore, studies have shown that dopaminergic (DAergic) input to the PFC increases during adolescence (8, 11, 12).

In addition to the above-mentioned increase in vulnerability towards drug-use and alcohol consumption, alcohol consumption during adolescence has been suggested to affect the normal brain development. MRI studies revealed an attenuated white matter growth and an accelerated gray matter reduction in heavy-drinking adolescents (13). Also, a previous study performed by DeWit and colleges shows that individuals with alcohol-debut during adolescence are at significant higher risk for developing alcohol use disorders (AUD) later in life (14).

Impairment of prefrontal function (e.g. impulse-control and decision-making) is an important feature of addiction (15). Sub regions of the PFC are activated during intoxication, craving and bingeing, and deactivated during withdrawal from drug-use (16). Using an attentional set-shifting task that depends upon the PFC for optimal performance, electrophysiological field recordings in the PFC of adult mice, has linked behavioral changes (i.e. deficits in cognitive flexibility) to changes in glutamatergic transmission (17). Also, age-dependent differences regarding basal glutamate levels and the frequency of spontaneous glutamate transients in the medial PFC of rats, have been demonstrated by Mishra et. al, using enzyme-based microelectrode amperometry. In particular, basal glutamate levels decreased with age and the frequency of spontaneous glutamate transients was higher in adolescent compared to adult rats (18). In juvenile rats, EtOH induces an inhibition of glutamatergic neurotransmission via the postsynaptic N-methyl-D-aspartate receptor (NMDAR) (19). However, recent studies now suggest that glutamatergic neurotransmission might also be inhibited in a presynaptic manner (20-22).

Much evidence suggest that the nicotinic acetylcholine receptor (nAChR) is the

converging site of action for nicotine and EtOH interactions (23), and these receptors have indeed been shown to prevail on GABAergic (γ-amino-butyric acid) interneurons in the PFC (24). Thus, it may be possible that EtOH interferes with the normal development of the brain and modulate the activity of the immature PFC, by enhancing GABAergic inhibition of glutamatergic synaptic transmission via the nAChR.

Therefore, the aim of this project was to analyse, at the synaptic level, if differences exist in the PFC of young rats compared with adult regarding glutamatergic neurotransmission – under normal conditions and following treatment with EtOH. We also aimed to investigate the potential role of nAChRs in this regard, using the nAChR antagonist mecamylamine (MEC).

The reward system

In the 1950's, Olds and Milner discovered that rats implanted with brain electrodes would repeatedly press a lever in order to receive electrical stimulation in some areas of the brain (25). These areas were referred to as "reinforcing structures" and were subsequently mapped anatomically and defined as the brain reward system (BRS) (26, 27). Brain areas associated with reward are the PFC, hippocampus, amygdala, nucleus accumbens (nAc), substantia nigra (SN) and the ventral tegmental area (VTA). The mesocorticolimbic system is defined as a neural pathway that connects the VTA with the ventral striatum (nAc and the olfactory tubercle) and the PFC (fig.1) (28). DAergic projection neurons within the mesocorticolimbic system, projecting from the VTA to the nAc, form the mesolimbic dopamine (DA) system, which is regarded as the core part of the BRS. Most drugs of abuse activate this part of the reward system

and cause DA release in the nAc (29), currently believed to convey their rewarding effects. The BRS is normally activated when what just happened was productive, i.e. when it yielded some a survival benefit. The activation of the BRS will also help the individual remember the circumstances and the behavior leading to such survival benefit in order to use it whenever required in the future. Following repeated use an addiction may develop as a consequence of reinforcement of maladaptive behaviors, resulting in e.g. change of priorities, irrational behavior and a loss of behavioral control. Areas of the brain associated with reward play different roles in the development of an addiction, which can be roughly divided into three stages. The binge/intoxication stage, the withdrawal/negative affect stage and the preoccupation/anticipation (craving) stage (30) (see "the development of an addiction", p. 11)

Addiction

Alcohol use

Alcohol addiction is a chronically relapsing disorder characterized by the compulsion to seek alcohol, loss of control in limiting intake and emergence of a negative emotional state (withdrawal syndrome), e.g., dysphoria, anxiety and irritability, when access to alcohol is prevented (31). In the current Diagnostic and Statistical Manual of Mental Disorders (DSM), DSM–5, published by the American psychiatric association (APA) in May 2013, alcohol use disorder (AUD) is a criterion diagnose based on clusters of symptoms. At least two out of eleven symptoms (fig. 2) must be present during the same 12-month period in order to meet the diagnose. The AUD diagnose is

further divided into three degrees of severity (mild, moderate and severe) depending on how many criteria are met.

DSM-5 merges two previous diagnoses from DSM-IV; alcohol dependence and alcohol abuse (32). Alcohol abuse, although no longer a psychiatric diagnose, refer to the continued consumption of alcohol despite adverse consequences. Alcohol dependence refers to the psychological and physiological elements of continued alcohol use, e.g. impaired control over drinking (psychological) and tolerance and withdrawal symptoms (physiological) (33).

The development of an addiction – three stages

Throughout the development of an addiction, impulsivity is a trait often dominating the early stages, associated with positive reinforcement mechanisms, while compulsivity (associated with negative reinforcement mechanisms and automaticity) combined with impulsivity impacts the later stages (for definitions, see table. 1) (30).

The first stage is the binge/intoxication stage. This stage is characterized by impulsive drug intake and positive reinforcement (table 1). Much emphasis has been put on the role of the mesolimbic DA system in mediating reward. Activation of these pathways result in wanting and desire (34) promoting goal-directed behavior and drug-seeking (35). However, much evidence now suggests that reinforcement occur independent of dopamine at the nAc. E.g., despite inactivation of the mesocorticolimbic dopamine system, rodents continue to show rewarding effects of alcohol and nicotine (36, 37). Multiple inputs to the activation of reinforcement in these brain regions are therefore indicated. The acute reinforcing effects of drugs of abuse seem to be mediated

primarily via the nAc with support from the central amygdala (CeA) and the ventral pallidum (VP).

In the second stage – the withdrawal/negative affect stage, the motivation for drug seeking comes from avoiding the negative emotional state, i.e. the withdrawal symptoms. Activation of the extended amygdala may be involved in conveying this negative emotional state. The acute withdrawal (table 1) is associated with neuroadaptation within the mesolimbic DA system (30). I.e., a decrease in activity of the mesolimbic DA system (38). Also, the acute withdrawal is associated with the recruitment of neurotransmitter systems such as dynorphin and corticotrophin that convey anxiety and stress (30). Anxiety-like behavior from EtOH withdrawal was reversed by intracerebroventricular administration of CRF1/CRF2 antagonists and by systemic administration of CRF1 antagonist. These effects have been localized to the CeA (39, 40). EtOH withdrawal is associated with increased dynorphin in the amygdala (41).

The third stage of the addiction cycle – the preoccupation/anticipation (craving) stage, is characterized by an increase in drug craving, triggered by an enhanced sensitivity to conditioned cues. In this stage, the individual reinstates drug-seeking behavior after abstinence (table 1) (30). The reinstatement of drug-seeking behavior can be induced by: the drug itself, drug conditioned cues, acute stressors or a residual negative emotional state after withdrawal. These reinstatements are mediated through different circuits and involve the PFC, nAc, VP and the amygdala (30, 42, 43).

The addiction cycle is composed by neuroadaptions in different brain circuits. Amongst the circuits hypothesized to be involved are the mesolimbic DA system and the dorsolateral /inferior frontal cortex /hippocampus circuits (30). The PFC is crucial for the development of an addiction. E.g., frontal cortex gray matter volumes, and preference for immediate gratification during decision-making, were inversely correlated in a study performed by Bjork et. al, 2009 (30, 44). Also, signs of regional brain damage and cognitive dysfunction can be observed in individuals addicted to alcohol even without any associated neurological or hepatic problems (45).

The prefrontal cortex

The cerebral cortex is composed out of six layers, the most external layer is layer I, and the most internal, layer VI. Each layer contains a characteristic distribution of neuronal cell types and connections. Layers II and III contain small to medium size pyramidal neurons and layers V and VI contains large pyramidal neurons (layer V containing most of them).

The rodent PFC can be subdivided into the orbitofrontal, the dorsomedial and the ventromedial cortex. The rodent PFC, as opposed to the primate PFC, is agranular, meaning that it lacks the granule-cell layer (layer IV) of the human PFC. However, within layer III lie thalamo-cortical fibers that (despite the lack of the granule-cell layer) demarcates the pyramidal neurons of the supra-granular layers II-III from the pyramidal neurons of the infragranular layers V-VI (46-48).

The PFC contains several different neuronal subtypes, which can be roughly divided into two major groups: pyramidal neurons (excitatory glutamatergic afferents) and

interneurons (inhibitory GABAergic neurons). The pyramidal neurons make up about 80% of the PFC neurons and the GABAergic neurons, about 20%.

The PFC layers V and VI receive glutamatergic afferents from other cortical areas and the thalamus, mainly to pyramidal neurons but also to interneurons. The basal forebrain provides cholinergic and GABAergic afferents, mainly projecting to layers V and VI although a small fraction project to Layers I-III (49). The main cholinergic input provides the whole cortical mantle with afferents and originates in the nucleus basalis of Meynert of the basal forebrain (BF) (50). The pyramidal neurons located in layers V and VI also receive projections from VTA DA neurons (28) (fig.3).

Interneurons are present in all cortical layers and can be functionally divided into two groups; fast spiking (FS) neurons and non-fast spiking (NFS) neurons. Pyramidal neurons are tonically inhibited by FS neurons while NFS neurons are involved in modulating the activity of FS neurons (28). Layer II and III pyramidal neurons project intracortically and synapse with other pyramidal neurons and GABAergic interneurons.

Layer V and VI pyramidal neurons project to layers II and III and provide subcortical areas including the VTA, thalamus and NAc with glutamatergic afferents (28) (fig. 3).

Ethanol and the nicotinic acetylcholine receptors

EtOH directly modulates the function of several types of receptors and ion-channels. While EtOH has a potentiating effect upon the functions of $GABA_AR$, $5\text{-}HT_3R$, GlyR and nAChR (51-53), the function of NMDARs is inhibited (54). The effect of alcohol upon these receptors and ion-channels are dependent on different variables, e.g. sub-unit composition of the receptor/ion-channel and alcohol concentration. As the main focus of this project is the involvement of nAChRs in the effects of EtOH, further discussion will be focused on this receptor system.

Receptors that are activated by the endogenous agonist acetylcholine (ACh) are termed cholinergic. Two main subtypes of cholinergic receptors are described: the muscarinic ACh receptor and the nicotinic ACh receptor (nAChR). As opposed to the muscarinic ACh receptor, the nAChR is activated by nicotine (the addictive ingredient of tobacco) giving this subtype its name. nAChRs can be further subdivided into neuronal nAChRs and muscle-type nAChRs. In this report we will be referring only to the neuronal subtype.

Twelve different subunits ($\alpha2–\alpha10$ and $\beta2–\beta4$) make up the different, pentameric nAChR subtypes, which can be either homo- or heteromeric in composition. The subtypes are named after their composition. E.g., the most common neuronal subtypes are named $\alpha4\beta2$ and $\alpha7$ (55). All subtypes have a central pore permeable to cations, mainly Na^+ and K^+. Some subtypes are also permeable to Ca^{2+} (56).

Neuronal nAChR can be present both pre- and postsynaptically. Pre-synaptically they can influence the release of neurotransmitter since some subtypes, as mentioned

above, are permeable to Ca^{2+}. In the PFC, interneurons of all layers contain nAChRs (57).

AIM

Aim

The overall aim of the project is to analyze, at the synaptic level, what is different in the PFC of young rats compared with adult rats. By designing experiments aimed at describing these differences in glutamatergic neurotransmission, both under normal conditions and following treatment with EtOH or EtOH + MEC in young and adult rats, it will ultimately be possible to dissect and hopefully understand the mechanism explaining why and how alcohol in early age increases the risk of alcohol problems later in life, and the role of nicotine in this regard.

Scientific issue

To begin dissecting the mechanism underlying age-dependent differences in glutamatergic neurotransmission in the prefrontal cortex this proposal is based on a few very basic questions:

- How is glutamatergic transmission different in the prefrontal cortex in young and adult animals?
- How does ethanol interact with and affect glutamatergic transmission depending on the age of the animal?
- Which synaptic mechanisms are responsible and how do they change with age?

MATERIALS AND METHODS

Electrophysiological experiments

In extracellular field potential recordings, stimulation electrodes are positioned in order to activate presynaptic glutamatergic afferents. This results in an influx of Na^+ and efflux of K^+ through glutamatergic receptors on the post-synaptic neurons. The net flow of positive charge is inward, creating a shift in electric potential – a population spike (PS) (fig. 4). This PS is reflecting the synaptically induced firing of action potentials and therefore, it can be classified as a type of field excitatory postsynaptic potentials (EPSP). Using extracellular recording electrodes, the shift in electric potential can be measured.

Drugs and solutions

Drugs and chemicals used in the experiments of this project are listed alphabetically below. All drugs were diluted in artificial cerebrospinal fluid (aCSF) to final concentrations. All drugs and solutions were aerated with carbogen (95% O2/ 5% CO2 gas).

aCSF	Content (in mM): 124 NaCl, 4.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 10 glucose, 2 $CaCl_2$
EtOH	Final concentrations: 20, 50 and 100 mM

Mecamylamine	nAChR antagonist. Final concentration: 10 μM.
Modified aCSF	Content (in mM): 194 sucrose, 30 NaCl, 4.5 KCl, 1$MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 10 glucose
NBQX	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antagonist. Final concentration: 5 μM

Brain slice preparation

Coronal slices of the PFC were prepared using male Wistar rats divided into the following age groups: juvenile (postnatal day (PND) 21-25, n = 9) and adult (PND > 60, n = 10) (18). Animals were bred at the University of Gothenburg, and maintained in a temperature and humidity controlled facility on a 12:12-h light/dark cycle (lights on at 6 AM). The animals had free access to food and water. Animals were anaesthetized with Isoflurane (Baxter medical, Kista, Sweden) and decapitated. The brains were rapidly dissected and placed in ice-cold aerated modified aCSF for at least 30 seconds of recovery. The brains were then glued (Loctite super glue) onto an ice-cold Teflon pad, cut into two hemispheres and mounted on a Vibratome (Leica VT 1200S, Nussloch, Germany), submerging the brains completely in aerated aCSF. Coronal slices of the PFC, 400 μm thick, were prepared and placed in aerated aCSF at room temperature for at least one hour of recovery (58). The slices were then transferred to recording chambers connected to a pump, allowing the brain tissue to be continuously perfused (at a constant rate of approximately 2.5 ml/min) with aerated aCSF or aCSF and drugs (MEC (10 µM), NBQX (5 µM) and EtOH (20, 50

and 100 mM)) warmed to 32°C using a water chamber. Spare slices were kept in room temperature aerated aCSF for a maximum of 5 hours for later use.

Field potential recordings

A recording electrode filled with NaCl (1M) (glass microelectrode, pulled on a pipette puller, Sutter instrument co., model P-97, Novato, CA, USA) was placed in the deeper layers (presumably layers V-VI) of the PFC close to the anterior part of the corpus callosum. Two stimulation electrodes (monopolar tungsten electrode, World precision instruments Inc., Sarasota, FL, USA) were placed ventrally of the recording electrode (fig. 5). The intensity of the electric current stimuli (approximately 40-60 mA) was set to yield PS amplitudes approximately half the size of the maximal evoked response. Stimulation occurred every 20 s from each electrode separated by 10 s. Once stable baseline responses were achieved (< 15 % variation of PS amplitudes) drugs were bath applied via the perfusion system. Antagonists were applied 20 minutes prior to treatment with EtOH and remained in the bath during the rest of the experiment. Signals were amplified using a custom made amplifier, filtered at 3 kHz, digitized at 8 kHz. The signals were then transferred to a PC for analysis.

Data analysis

Data was analyzed using Clampex 10.1 (Molecular Devices, Foster City, CA). Graphs were assembled using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). In text, data are presented as mean values at t = 26-30 minutes compared to individual baseline calculated at t = 6-10 minutes with 95 % confidence interval (CI). In time course figures, each data point is a mean value of 3 analyzed PSs recorded over 1

minute, plotted as mean PS amplitude compared to baseline with standard error of the mean (SEM). Two-way ANOVA was used for statistical analysis. Bonferroni's multiple comparisons test was used for post-hoc analysis when applicable. An unpaired t-test was used to analyze the effect of MEC on PS amplitude. Data used for statistical analysis was grouped based on treatment and/or age (table 2), and was collected from the electrophysiological experiments.

Methodological considerations

An advantage using this method is that large populations of cells are intact, allowing us to study the effect of specific receptor-systems on the field potential. Although the results of this method may not reflect what happens in the live animal (since axons are cut) it does reflect what happens in a large population/system of cells, compared to single-cell patch-clamp recordings, reflecting the events in one cell at a time. One disadvantage using this method is the difficulty in determining the order of events. However, EtOH can increase the release-probability for some neurotransmitters and decrease release-probability for others. Using field potential recordings, the net effect of EtOH on PFC output can be evaluated.

Ethics

All efforts were made to reduce the number of animals used throughout the project. Animals were handled with care and sedation was performed via inhalation, thus not causing the animals physical pain due to injections. The experiments have been approved by Göteborgs djurförsöksetiska nämnd (N266/12).

RESULTS

No significant difference was observed between the baseline PS amplitudes in any of the statistical analysis performed (data not shown).

Characterization of response

NBQX strongly inhibited the second negative inflection (i.e. PS amplitude) of the electrically evoked field potential

The amplitudes of the first (N1) and the second (N2) negative inflections (after the stimulus artifact) of the electrically evoked field potentials in the PFC were measured. When brain slices were treated with AMPAR antagonist NBQX (5 μM), the amplitude of N2 was initially increased (peak PS amplitude = 188.8 % of baseline after 4 minutes of treatment) and then strongly inhibited (PS amplitude = 31% of baseline after 9 minutes of treatment and throughout the experiment). N1 remained unaffected; strongly indicating that N2 is postsynaptic and mainly mediated via AMPAR while the N1 is presynaptic (fig 6A, 6C). Throughout this report N2 will be referred to as PS amplitudes and will always be measured at the lowest point of the second negative inflection.

The electrically evoked field potential is a physiological and dynamic response

At the beginning of the recording sessions, a stimulus/response (input/output) curve was set up for a group of slices. We measured PS amplitudes during increased stimulation intensities (μA) and found that higher stimulus strength resulted in larger PS amplitudes (mV) of both the pre- (N1) and postsynaptic component (N2). Decreasing the stimulus strength reversed this effect and the PS amplitudes went back to baseline-level (fig. 6B), strongly indicating a physiological response.

Ethanol depresses PFC output

Acute treatment with EtOH induced a depression of PS amplitudes evoked in the PFC of juvenile brain slices. Statistical analysis using two-way ANOVA revealed significant effect on time (F_{time} (1,23)=13.01, $p < 0.05$) but no significant effect on treatment and no interaction effect. Post hoc analysis of the time-effect using Bonferroni´s multiple comparisons test showed that treatment with 50 mM EtOH induced a significant depression of PFC output (PS-amplitude = 86 ± 4.7% of baseline, $p < 0.01$, n = 12), as did treatment with 100 mM EtOH (PS amplitude = 86 ± 4.4 % of baseline, $p < 0.01$, n = 9). There was however, no significant effect on PS amplitudes induced by treatment with 20 mM EtOH (PS amplitude = 95 ± 0.5% of baseline, n = 4) (fig. 7A, 7B). There was no significant difference between the 50 mM and 100 mM EtOH groups.

Ethanol-induced depression of PFC output is blocked by mecamylamine

Statistical analysis, using an unpaired t-test, on the effect of treatment with nAChR antagonist MEC (10 μM), did not reveal a depression of PS amplitude of drug free baseline (fig. 8A). Further investigating the effect of EtOH on PS amplitude in the presence of MEC, statistical analysis using two-way ANOVA revealed significant effect on interaction ($F(1, 17) = 11,1$, $p < 0.01$) and drug treatment ($F(1, 17) = 10,86$, $p < 0.01$) but no effect on time . Post hoc analysis using Bonferroni´s multiple comparisons test showed that the ethanol-induced depression of PS amplitude was blocked by MEC (10 μM) (PS amplitude = 86 ± 4.4% of MEC treated baseline, $p < 0.0001$) (fig. 8B, 8C).

Ethanol significantly depresses PFC output in juvenile, but not adult rat slice preparations

Treatment with 50 mM EtOH induced a depression of PS amplitude in juvenile rat brain slice preparations (fig. 7A, 7B). Statistical analysis, using two-way ANOVA, revealed no significant depression of PS amplitude in the adult rat brain slice preparations. There was, however, no significant difference in PS amplitudes between the juvenile and adult groups (fig. 9A, 9B).

DISCUSSION WITH CONCLUSIONS

The present study demonstrates that EtOH inhibits glutamatergic synaptic transmission in the PFC of juvenile rat brain slices. In addition, the observed, inhibiting effect was blocked by nAChR antagonist MEC, indicating it may be mediated via nAChRs on GABAergic neurons. We also demonstrate that EtOH inhibits glutamatergic synaptic transmission in the PFC of juvenile, but not adult, brain slices in a significant manner.

During characterization of the evoked field response, the PS amplitudes of brain slices treated with AMPAR antagonist NBQX (5 μM) were initially increased and then blocked. A possible explanation could be that AMPAR present on GABAergic interneurons were blocked earlier, or to a higher extent, than those present on glutamatergic neurons. That would result in an initial loss of net inhibition of glutamatergic synaptic transmission. As the time of treatment with the antagonist passed, a larger percentage of AMPAR were blocked (including those present on the glutamatergic neurons), leading to the observed inhibition of glutamatergic neurotransmission (fig. 6C).

In this study, we showed that PS amplitudes evoked in the juvenile PFC were significantly depressed by 50 mM and 100 mM EtOH (fig. 7A, 7B). EtOH-sensitive AMPA responses have been reported in other brain regions, e.g. the nAc (21), central amygdala (59) and the CA3 of the hippocampus (60). These studies declare a depression of AMPAR-mediated responses, while an electrophysiological whole-cell study performed in the somatosensory cortex of juvenile rat brain slices (61) found that AMPAR-mediated transmission was depressed by EtOH in some brain slices, but potentiated in others. However, the observed potentiation occurred in brain slices of very young rats (PND < 9), and the depression in rats age PND > 15, which is more comparable to the age of the juvenile rats of our experiments (PND 21 - 25).

Whole-cell recordings in the medial PFC of juvenile rat brain slices did not show a significant depression by EtOH of AMPAR mediated excitatory post synaptic currents (EPSCs), even at concentrations which could be fatal to a non-tolerant human individual (88 mM – corresponding to approximately 3.3 $^{0}/_{00}$ in plasma) (19). Although they did not observe significant depressions of AMPAR-mediated neurotransmission by EtOH, the discrepancies between our studies might be due to the fact that our methods differed. Whole-cell patch clamp recordings allow measurement from only one neuron at a time, while the present study measured evoked field potential amplitudes, representing the net effect upon output in populations of cells. Another difference regarding our methods is that their recordings were made at lower temperatures (25-28℃ compared to our 32℃). Future work will be required to establish the exact reasons for the discrepancies between these studies.

In this study, we observed a small depression of PS amplitude by treatment with 20 mM EtOH in the juvenile slices. This effect was, however, not significant after statistical analysis. This result is based on relatively few experiments (n = 4) and it is possible that the depressive effect observed on PS amplitude, would be significant, had we had the possibility to perform more experiments using 20 mM EtOH.

There was no significant difference between the 50 mM and 100 mM EtOH groups. A possible explanation for this might be that the receptor-systems involved and modulated by EtOH was already fully occupied, according to the widely accepted receptor occupation theory, hence there would be no further pharmacological effect even though a higher concentration of EtOH was applied. The statistical analysis of the effect was performed at 16-20 minutes of treatment. Had we performed the analysis at an earlier time point, where the plots in time course figures were more separated from each other, a significant difference could have been observed. Taken together, it would be interesting to perform more experiments on juvenile brain slices to further evaluate if there is an actual dose-dependent depression of PS amplitudes by EtOH in the PFC. On the one hand, with previously studied concentrations (20, 50 and 100 mM) and on the other hand, with concentrations of EtOH somewhere in between 20 and 50 mM, and 50 and 100 mM respectively.

In the present study, the maximal depression was observed in treatment with 100 mM EtOH (a concentration correlated to heavy drinking in humans (19)) and measured approximately 17%. This finding is in agreement with other studies, reporting a depression of AMPAR-mediated responses by EtOH ranging from approximately 21% to 35% (21, 59, 60).

The observed inhibition of glutamatergic neurotransmission in juvenile brain slices in the present study, was blocked by nAChR antagonist MEC (fig. 8B, 8C). Studies performed in the rat neocortex, using immunocytochemical and patch-clamp methods, show that nAChRs prevail on both pyramidal neurons and interneurons in layer V of the PFC, and that nAChRs, present primarily on the GABAergic terminals, control GABA release onto the layer V pyramidal neurons (24). Furthermore, nAChRs have been shown to excite GABAergic neurons in the NAc (62) and EtOH has been postulated to potentiate nAChRs by stabilizing the open-channel state of the receptor (63). Taken together this strongly indicates that the depressant effect of EtOH is mediated via nAChRs, presumably by excitation of GABAergic interneurons (fig. 10). Future work will be required, however, to establish the role of GABAergic interneurons in the nAChR-mediated depression by EtOH of glutamatergic neurotransmission in the PFC.

We also found that EtOH inhibits glutamatergic synaptic transmission, in a significant manner, in juvenile, but not adult rat brain slices. Similar results were observed in a study performed in the nAc, showing that EtOH (50 mM) inhibits AMPAR-mediated glutamatergic synaptic transmission more strongly in the nAc of juvenile than of adult mice (20). However, studies performed in CA3 neurons of the hippocampus show that the AMPAR-mediated ESPCs in juvenile (PND = 23) slices were insensitive to EtOH while they were inhibited in very young rats (PND = 4) (60). Taken together these findings indicate an age-related, enhanced sensitivity towards EtOH-induced modulation of glutamatergic neurotransmission.

Possible mechanisms explaining why EtOH inhibits neurotransmission significantly of juvenile rat brain slices, but not adult, could be an age-related alteration in the distribution of nAChRs during brain development/aging or the loss of interneurons expressing these receptors. Moreover, there is a general deficit in cholinergic neurons in the forebrain during aging (64). However, if we had performed these experiments in larger groups, it is possible that there would be a significant depression of PS amplitude in the adult rat brain slices and a significant difference between the juvenile and adult groups.

The data from the present study further supports the notion that the adolescent brain is more sensitive to the effects of EtOH. Thus underscoring the importance of developing effective preventive interventions in order to postpone alcohol-debut as late as possible.

POPULÄRVETENSKAPLIG SAMMANFATTNING

ALKOHOL OCH ÅLDERSBEROENDE PÅVERKAN PÅ NERVSIGNALERING

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2017

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Alkohol har länge varit och är fortfarande en del av de flesta samhällen och kulturer, och intas bl.a. på grund av sin euforiska effekt. Bruk av alkohol kan dock också leda till sjukdom och ohälsa i form av t.ex. lever- och hjärt-kärlsjukdom, eller skador (avsiktliga och oavsiktliga). Det är inte heller bara den som brukar alkohol som kan ta skada av det. Familjemedlemmar, kollegor och vänner påverkas, och samhället påverkas med avseende på kostnader för sjukvård m.m. Alkohol är den tredje största riskfaktorn för mortalitet och sjukdom.

Nikotin är det beroendeframkallande ämnet i tobak. En stor mängd forskning har visat att det finns ett samband mellan alkohol-bruk med nikotin-bruk. Bland annat har forskning visat att de som börjar röka i tidig ålder löper en större risk för att hamna i alkohol-missbruk eller beroende senare i livet (6), och att ungdomar som börjar röka eller dricka alkohol löper en signifikant högre risk för att börja med det andra nära inpå (7).

Under ungdomsåren sker strukturella förändringar i hjärnan, framförallt i de delar av hjärnan som är associerade med framsteg i intellektuella funktioner, såsom t.ex. arbetsminne, impulskontroll (förmåga att motstå frestelser) och problemlösning (8). Ungdomsåren är dock också associerade med ett ökat risk-beteende och ett känslostyrt beteende, och det tros finnas ett samband mellan detta beteende och en ökad känslighet för belöning i hjärnan. En teori är att ungdomar får en större frisättning av dopamin än vuxna i hjärnans belöningssystem, vilket kan bidra till det att fortsatta utövandet av det beteende som gav upphov till belöningen, t.ex. att dricka alkohol eller röka (10).

Utöver den ökade sårbarhet som ungdomar har för att hamna i ett alkohol- eller drogmissbruk, så tror man även att bruket av alkohol i sig påverkar hjärnans normala utveckling. Forskning har visat att ungdomar som drack mycket alkohol hade en påskyndad strukturell förändring i hjärnan (13). Dessutom har alkoholdebut under ungdomsåren sammanlänkats med en ökad risk för alkoholmissbruk senare i livet (14).

Den här studien syftade därför till att undersöka om det finns skillnader i dessa områden av hjärnan mellan unga och vuxna råttor, med avseende på nervsignalering. Dels under normala förhållanden och dels efter behandling med alkohol. Dessutom ville vi undersöka om nervsignaleringen påverkades om man inaktiverade nikotinreceptorn (den del av nervcellen som har till uppgift att fånga upp och vidarebefordra signaler förmedlade via nikotin).

För att undersöka detta utförde vi mätningar av den skillnad som uppstår i elektrisk potential, när man stimulerar nervceller med ström, i skivor av unga och gamla råtthjärnor.

De huvudsakliga fynden var att alkohol dämpade nervsignaleringen i unga råtthjärnor och att denna effekt verkar vara förmedlad via nikotinreceptorer, troligen belägna på nervceller med just dämpande effekt. Alkohol dämpade nervsignaleringen signifikant i unga råtthjärnor, men inte vuxna.

Dessa fynd stödjer uppfattningen att ungdomars hjärna är mer känslig för alkohols effekter och understryker vikten av att utveckla effektiva preventiva åtgärder för att senarelägga alkoholdebut så mycket som möjligt.

ACKNOWLEDGEMENTS

To my supervisor, *Björn Schilström* – your guidance and support has been invaluable. What once seemed like an impossible task, I accomplished thanks to you! Thank you for your time and patience.

Louise Adermark, thank you for supporting us throughout this project, helping with everything from study design to analysis and interpretation of data.

Amir Lotfi, your laboratory expertize during the experiments of this project, has been very helpful. We can't thank you enough.

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TABLES, FIGURES AND APPENDICES

Tables

Table 1. Definitions

Positive reinforcement	The process by which presentation of a stimulus, usually pleasant, increases the probability of a response.
Negative reinforcement	The process by which the removal of an aversive stimulus, increases the probability of a response.
Impulsivity	Characterized by the failure to resist temptation and relief of the urge upon acting
Compulsivity	Characterized by anxiety and/or stress before committing a compulsive repetitive behavior and relief of anxiety/stress by preforming the compulsive behavior
Automaticity	Doing things without occupying the mind.
Withdrawal/abstinence	A group of symptoms that occur after abrupt decrease in drug intake or discontinuation.

Table 2. Number of experiments performed in each treatment and/or age group

Group	Number of experiments (n)
EtOH 20 mM	n = 4
EtOH 50 mM	n = 12
EtOH 100 mM	n = 9
Mecamylamine 10 μM + aCSF	n = 4
Mecamylamine 10 μM + EtOH 50 mM	n = 6
EtOH 50 mM (juvenile)	n = 12
EtOH 50 mM (adult)	n = 10

Figures

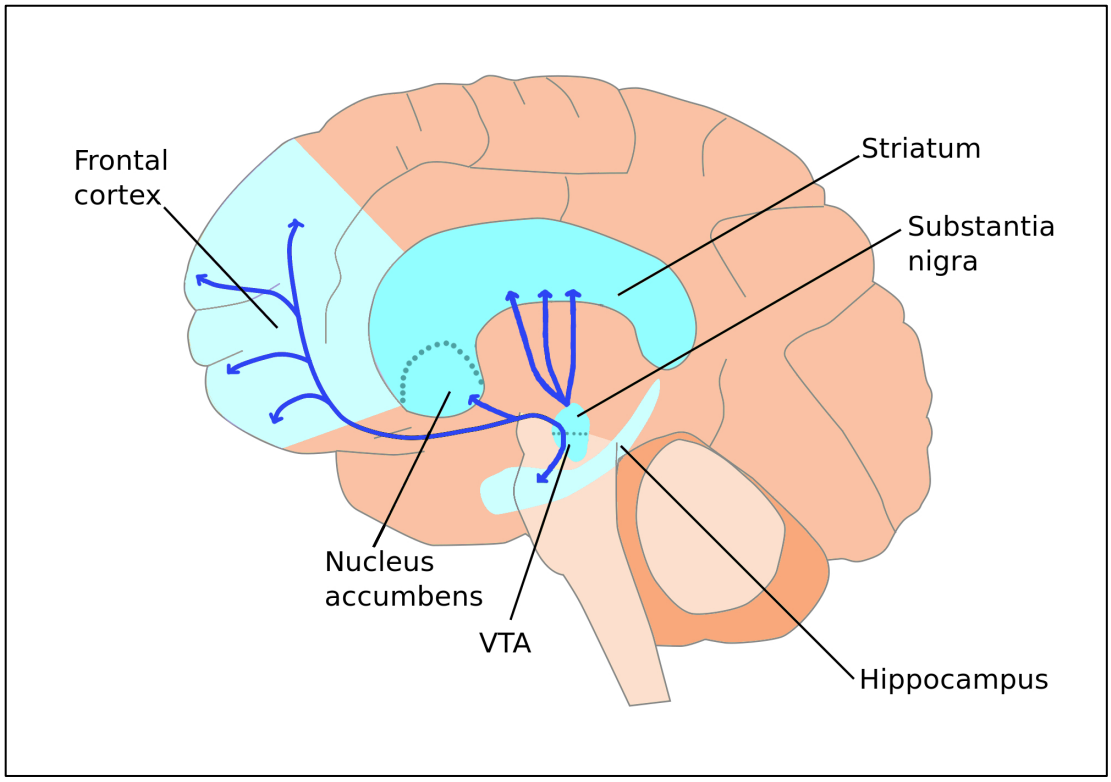

Figure 1. Schematic illustration of the mesocorticolimbic system, projecting from the VTA to the ventral striatum (nAc and olfactory tubercle) and the prefrontal cortex, and the nigrostriatal dopamine system projecting from Substantia nigra to the striatum. Image adapted from www.neojungiantypology.com

Figure 2. The alcohol use disorder (AUD) is indicated when an individual meets at least two of these criteria during a 12-month period. Depending on how many criteria are met, the AUD diagnose can be mild (2-3), moderate (4-5) or severe (6 or more). Image: DSM-5.

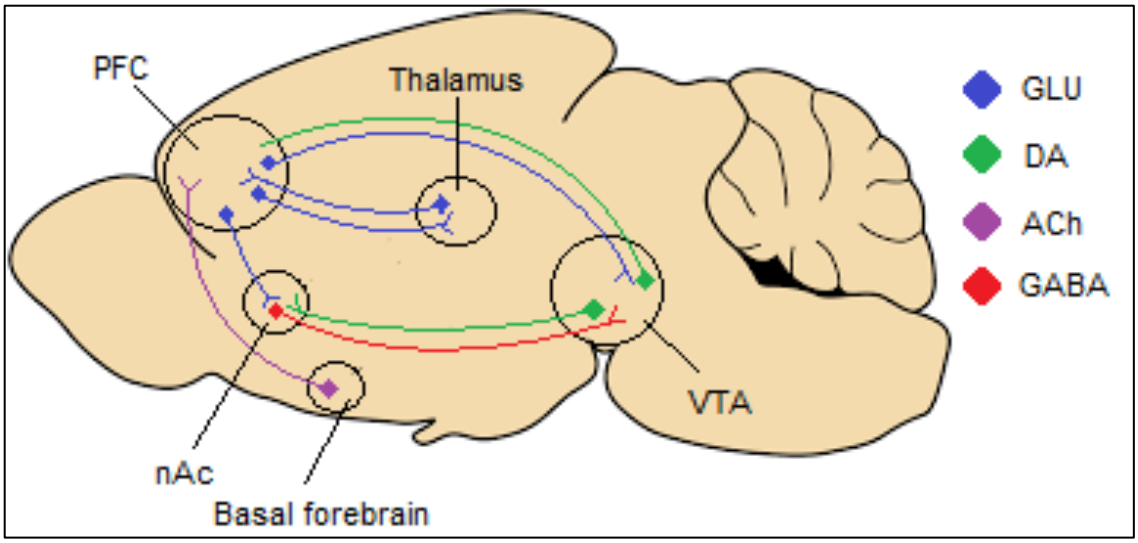

Figure 3. Schematic illustration of the DAergic (DA), Glutamatergic (GLU), GABAergic (GABA) and nicotinergic (ACh) projections to and from the PFC in the sagittal rodent brain. Image adapted from Wikimedia.org

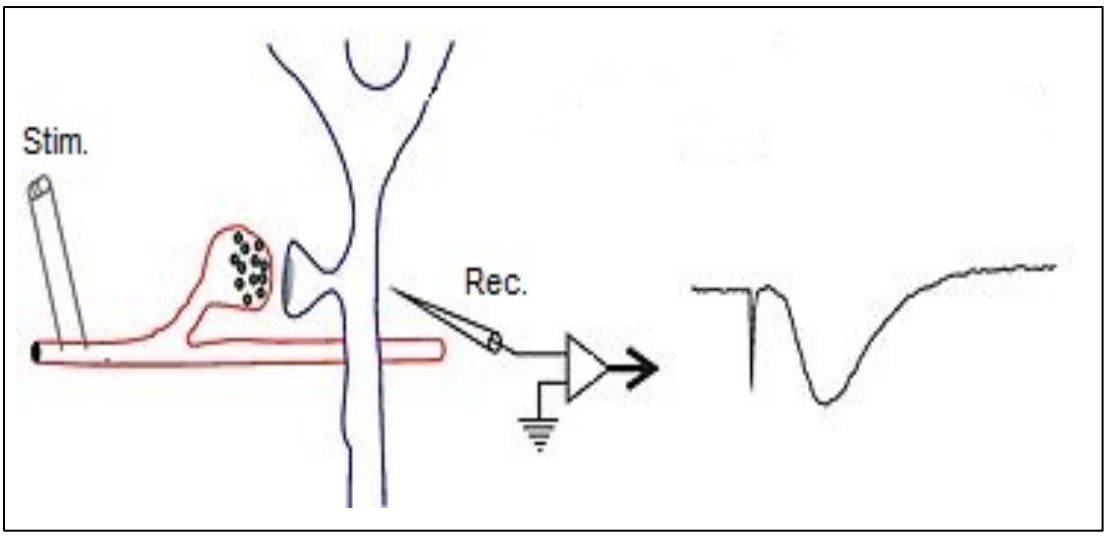

Figure 4. Schematic illustration of the principal behind extracellular field potential recordings. Stimulation (stim.) of the presynaptic terminal results in glutamate release onto the postsynaptic cell (this illustration is meant to represent a large population of synapses and neurons). Ionotropic glutamate receptor channels open and this results in an influx of Na^+ and efflux of K^+. The net flow of positive charge is inward and a recording electrode (rec.) placed in the extracellular space detects this as a current sink. Image adapted from wikipedia.org

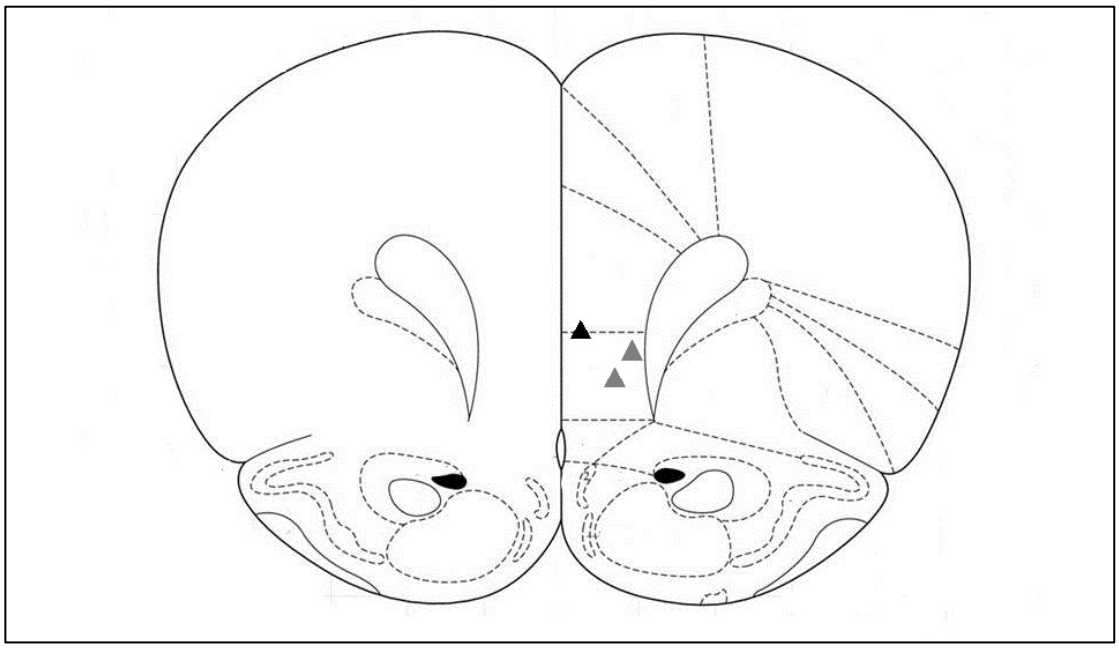

Figure 5. Schematic illustration showing the approximate placement of the stimulation electrodes (grey triangles) near the corpus callosum in the prelimbic/infralimbic areas of the prefrontal cortex, and the recording electrode (black triangle) placed in presumed layer V/VI of the prefrontal cortex. Image adapted from Paxinos, George, and Charles Watson. *The rat brain in stereotaxic coordinates,* Access Online via Elsevier, 2006.

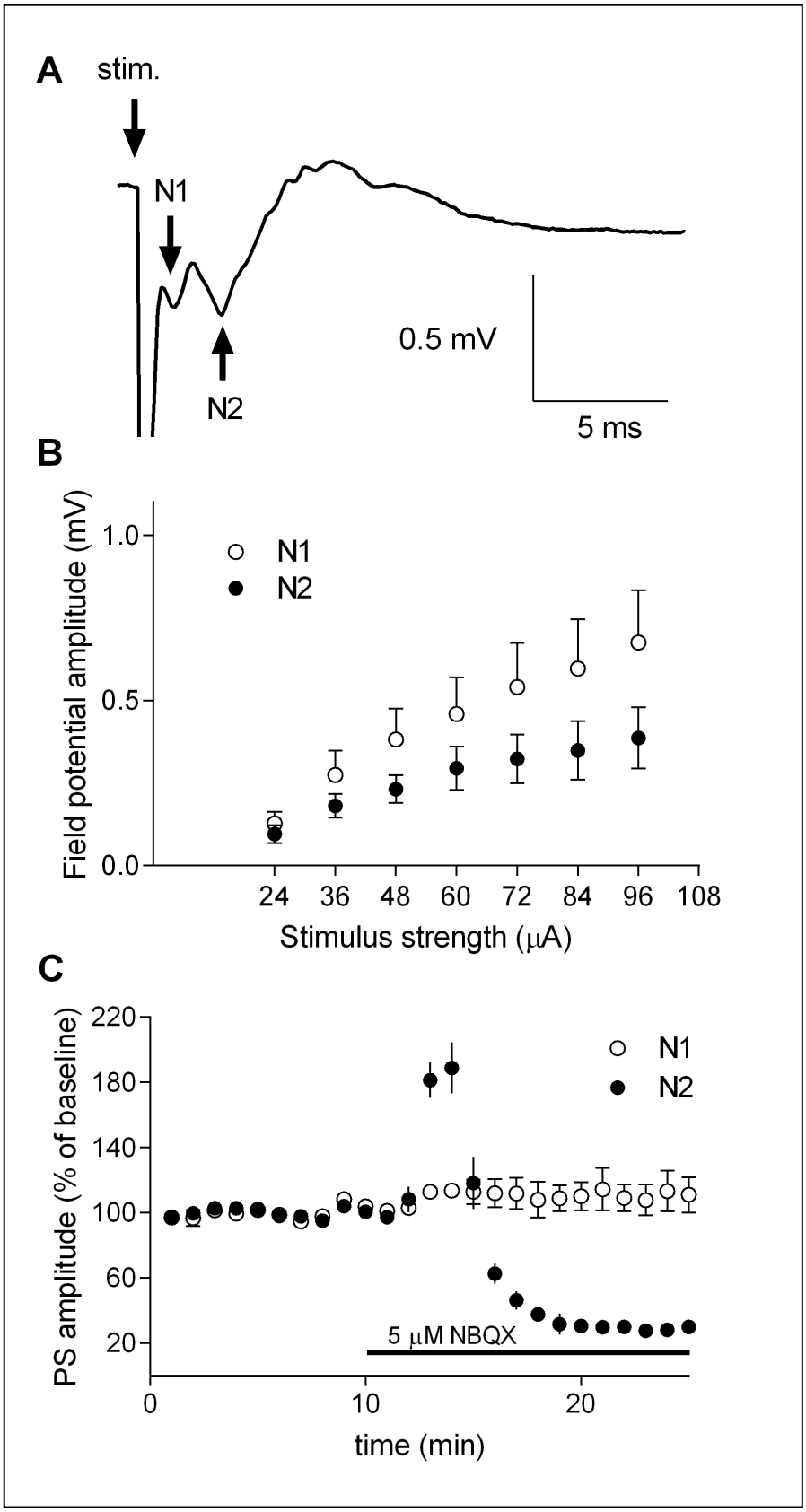

Figure 6. Characterization of response (A) The amplitudes of the first (N1) and the second (N2) negative inflections after the stimulus artifact (stim.) of the electrically evoked field potentials in the PFC were measured. (B) Higher stimulus strength (mA) resulted in larger amplitudes (mV) of both the pre- (N1) and postsynaptic component (N2). (C) The second negative inflection (i.e. PS amplitude) of the electrically evoked field potential was initially enhanced but then strongly inhibited by NBQX after approximately 5 minutes of treatment. Data show mean PS amplitudes compared to baseline with SEM.

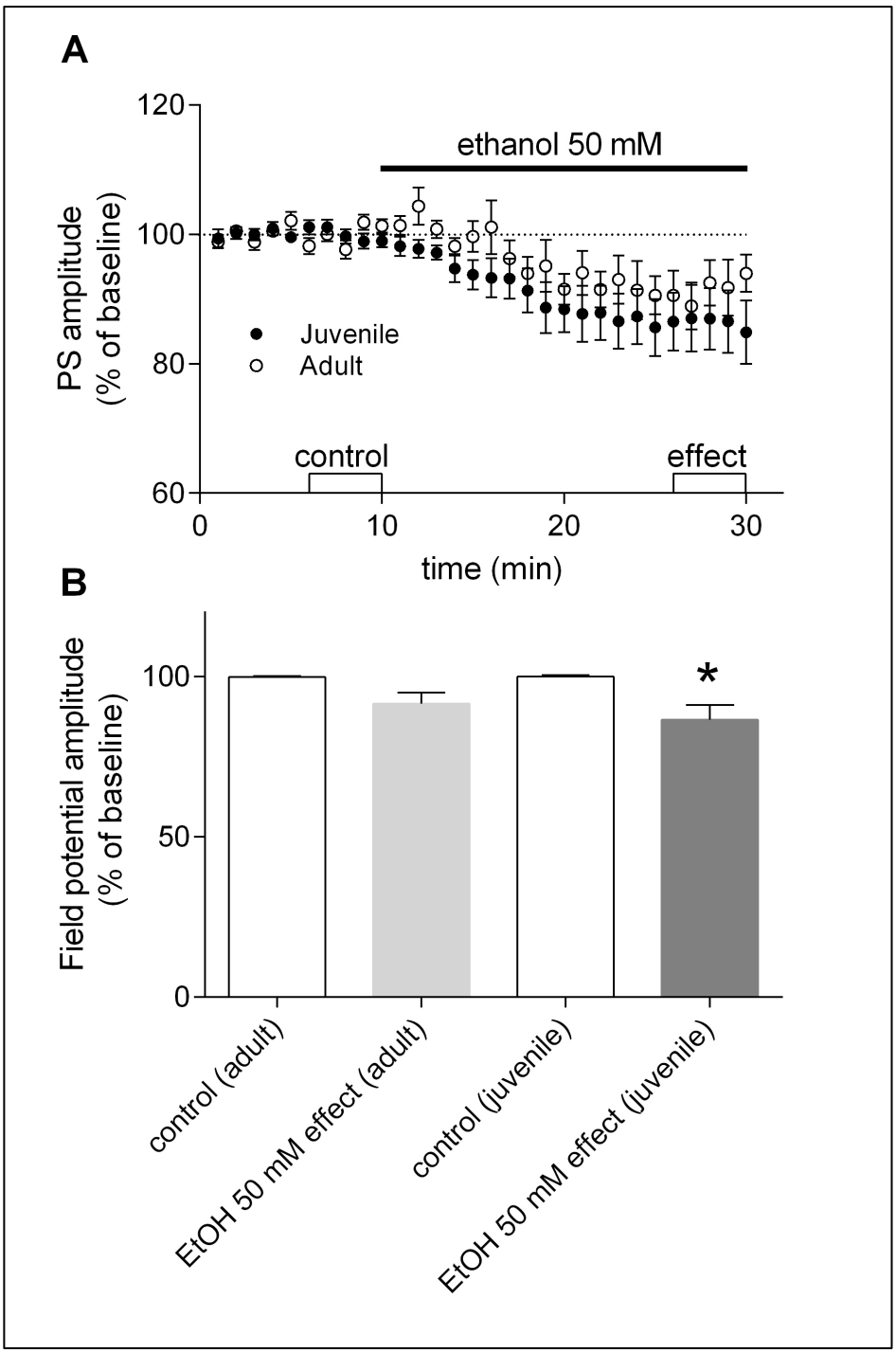

Figure 7. Acute treatment with ethanol (EtOH) depresses PFC output in juvenile rat brain slices (A) Treatment with 50 mM (n=12) and 100 mM (n=9) EtOH but not 20 mM (n=4) induced a statistically significant depression of PS amplitude ($p < 0.01$). There was no significant difference in PS amplitude between the 50 mM and the 100 mM group. (B) Bar graphs show change from baseline level before (control) and after (effect) treatment. Data show mean PS amplitudes compared to baseline with SEM. * $p < 0.05$.

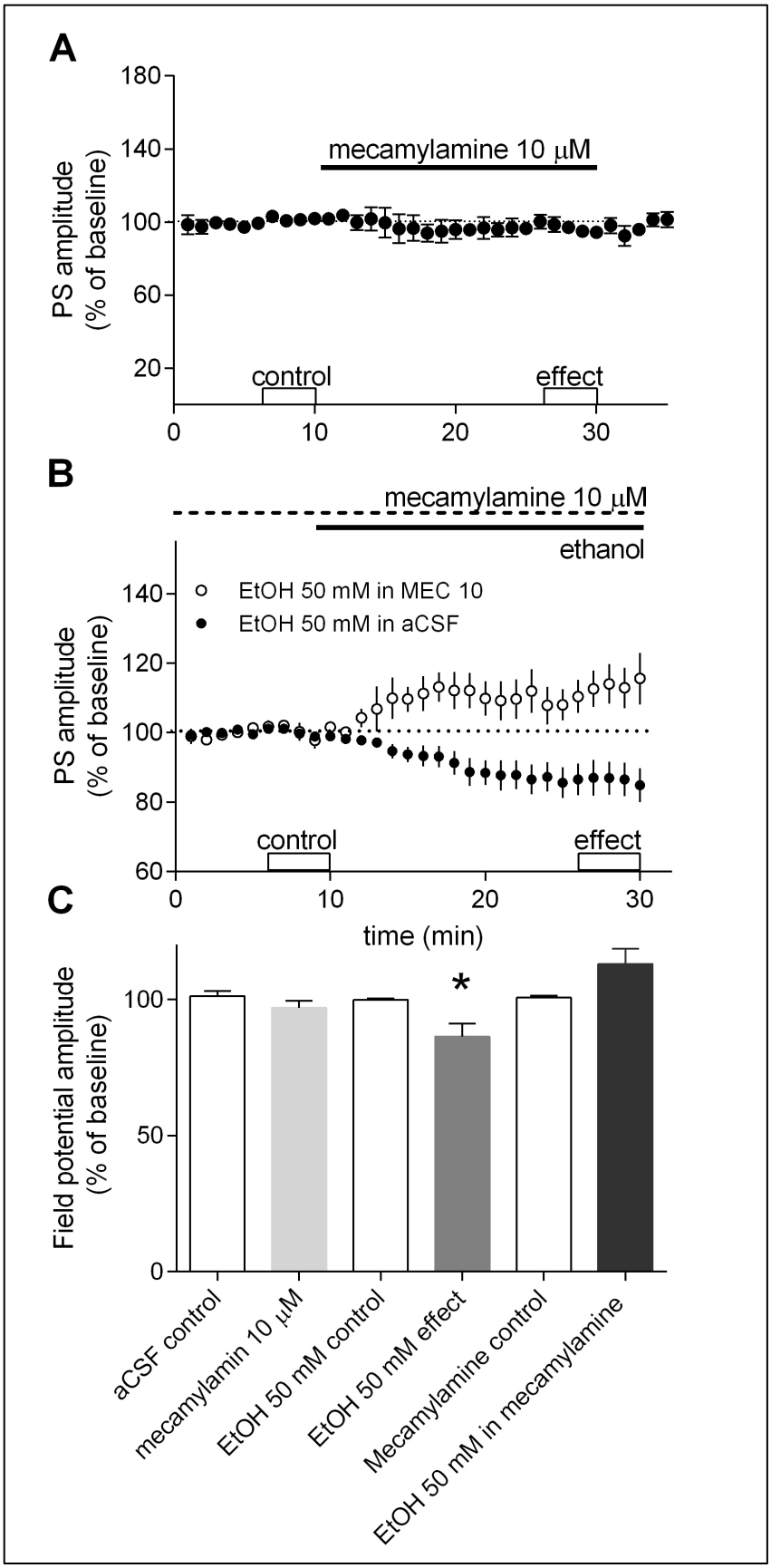

Figure 8. Ethanol (EtOH) -induced depression of PFC output is blocked by mecamylamine (A) Statistical analysis, using unpaired t-test, on the effect of treatment with mecamylamine (n=4) (10 μM), did not reveal a depression of PS amplitude of drug free baseline. (B) Further investigating the effect of EtOH on PS amplitude in the presence of mecamylamine, statistical analysis revealed significant effect on interaction and drug treatment ($p < 0.01$). Post hoc analysis showed that the EtOH induced depression of PS amplitude was significantly prevented by mecamylamine (n=6) (10 μM) ($p < 0.0001$). (C) Bar graphs show change from baseline level before (control) and after (effect) treatment with mecamylamine and/or EtOH. Data show mean PS amplitudes compared to baseline with SEM. * $p < 0.05$.

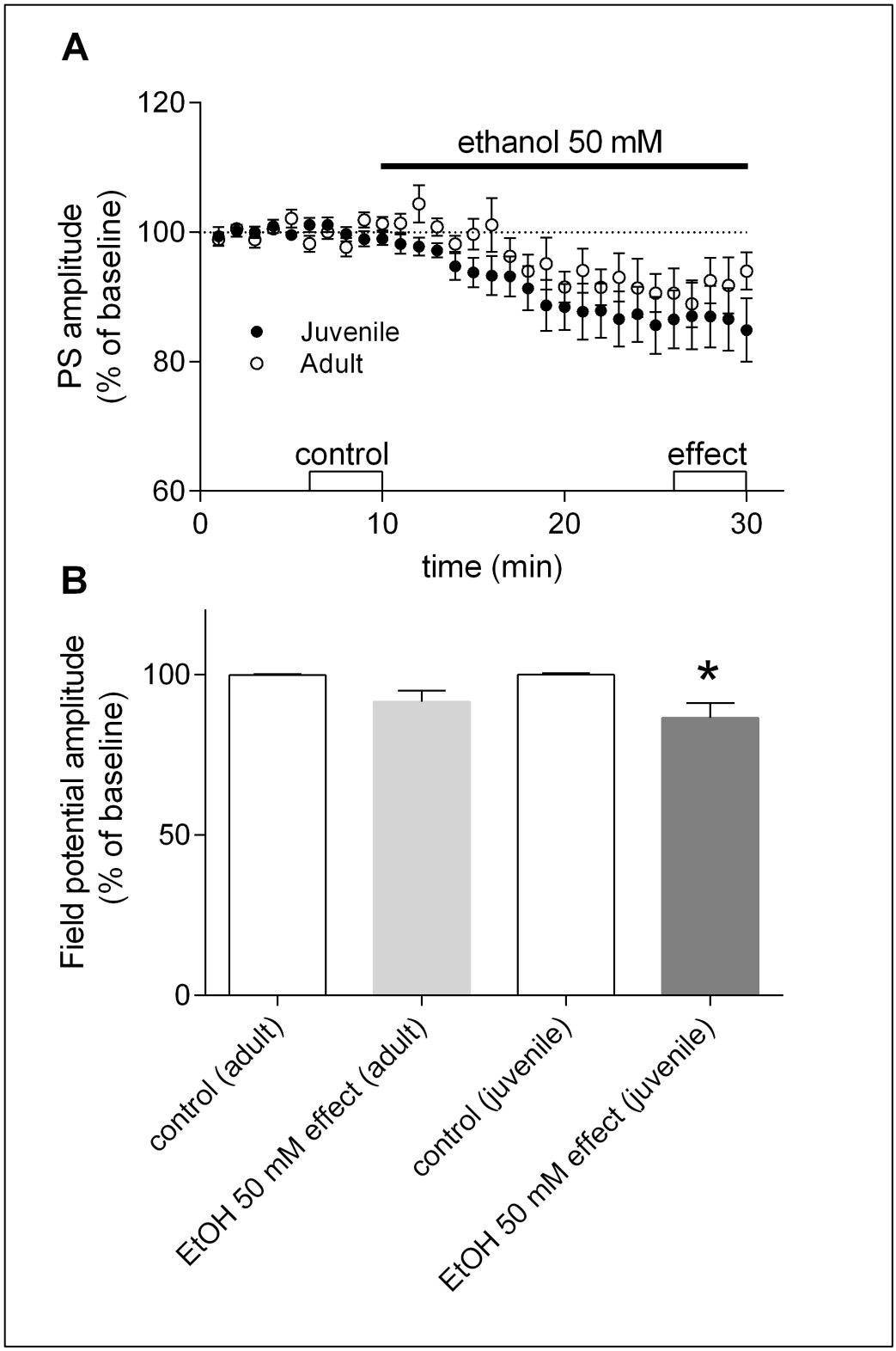

Figure 9. (A) Treatment with 50 mM ethanol (EtOH) significantly depressed PS amplitude in juvenile (n=12) but not in adult (n=10) rat slices preparations ($p < 0.01$). There was, however, no significant difference between PS amplitudes of the two groups. (B) Bar graphs show change from baseline level before (control) and after (effect) treatment in juvenile vs. adult rat brain slices. Data show mean PS amplitudes compared to baseline with SEM.* $p < 0.05$.

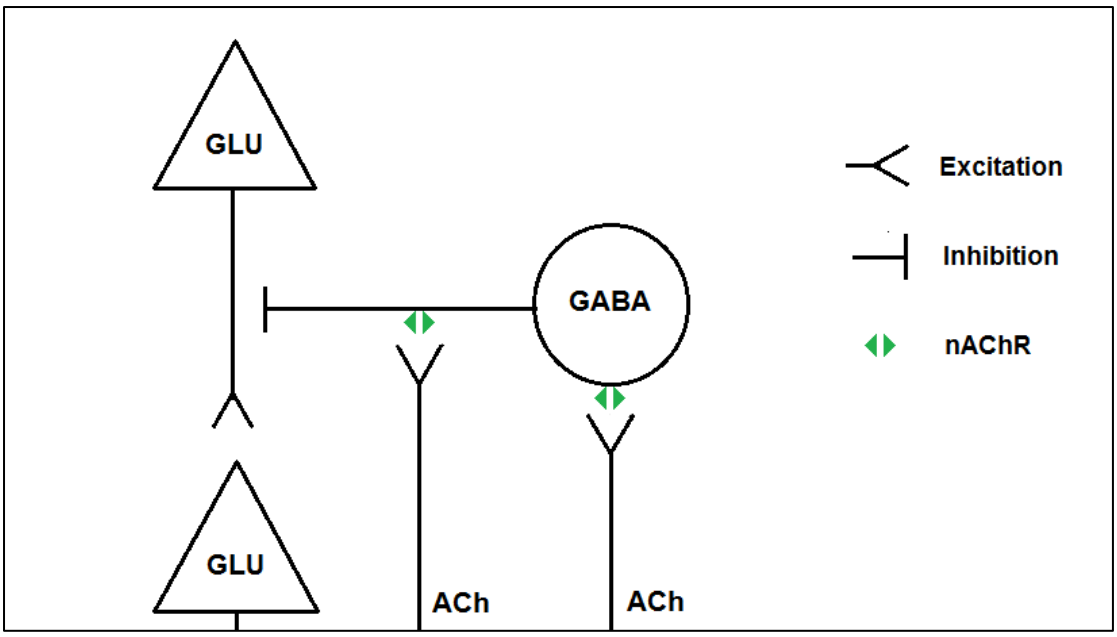

Figure 10. Schematic drawing showing how ethanol (EtOH) might influence prefrontal cortex (PFC) output by modulating the activity of nicotinic acetylcholine receptors (nAChR). Elevated levels of acetylcholine (ACh) drive excitation of GABAergic interneurons via the nAChRs, which in turn reduce glutamatergic neurotransmission, resulting in a net depression of PFC output.