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The Effects of Perinatal Choline Supplementation on Hippocampal Cholinergic Development in Rats Exposed to Alcohol During the Brain Growth Spurt

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Abstract

Prenatal alcohol exposure leads to long-lasting cognitive and attention deficits, as well as hyperactivity. Using a rat model, we have previously shown that perinatal supplementation with the essential nutrient, choline, can reduce the severity of some fetal alcohol effects, including hyperactivity and deficits in learning and memory. In fact, choline can mitigate alcohol-related learning deficits even when administered after developmental alcohol exposure, during the postnatal period. However, it is not yet known how choline is able to mitigate alcohol-related behavioral alterations. Choline may act by altering cholinergic signaling in the hippocampus. This study examined the effects of developmental alcohol exposure and perinatal choline supplementation on hippocampal M₁ and M_{2/4} muscarinic receptors. Sprague-Dawley rat pups were orally intubated with ethanol (5.25 mg/kg/day) from postnatal days (PD) 4-9, a period of brain development equivalent to the human 3rd trimester; control subjects received sham intubations. From PD 4-30, subjects were injected s.c. with choline chloride (100 mg/kg/day) or saline vehicle. Open field activity was assessed from PD 30-33 and brain tissue was collected on PD 35 for autoradiographic analysis. Ethanol-exposed subjects were more active compared to controls during the first two days of testing, an effect attenuated with choline supplementation. Developmental alcohol exposure significantly decreased the density of muscarinic M₁ receptors in the dorsal hippocampus, an effect that was not altered by choline supplementation. In contrast, developmental alcohol exposure significantly increased M_{2/4} receptor density, an effect mitigated by choline supplementation. In fact, M_{2/4} receptor density of subjects exposed to alcohol and treated with choline did not differ significantly from that of controls. These data suggest that developmental alcohol exposure can cause long-lasting changes in the hippocampal cholinergic system and that perinatal choline supplementation may attenuate alcohol-related behavioral changes by influencing cholinergic systems.

Keywords

muscarinic; fetal alcohol; treatment; hyperactivity; nutrition

INTRODUCTION

Fetal alcohol spectrum disorders (FASD) is a term that refers to a range of disruptions in physical and behavioral development, which vary in severity and pattern among children

whose mothers consumed alcohol during pregnancy (Mattson and Riley, 1998; Sokol et al., 2003; Riley and McGee, 2005). For example, prenatal alcohol exposure is known to cause hyperactivity and learning deficits that persist into adulthood (Bond, 1986; Uecker and Nadel, 1996; Mattson and Riley, 1998; Hamilton et al., 2003). Using a rat model, we have previously shown that the perinatal supplementation of the nutrient choline can reduce the severity of FASD, including attenuation of alcohol-related hyperactivity and learning deficits (Thomas et al., 2000; Thomas et al., 2004; Thomas et al., 2007; Ryan et al., 2008; Thomas et al., 2010). In particular, choline supplementation during the early postnatal period attenuates ethanol's adverse effects on behaviors that rely on the functional integrity of the hippocampus (Thomas et al., 2000; Wagner and Hunt, 2006; Ryan et al., 2008; Thomas and Tran, 2010).

Choline is a quaternary amine, classified as an essential nutrient (Zeisel and da Costa, 2009). It subserves various signaling pathways, as a precursor to sphingosyl-phosphocholine, diacylglycerol, and the neurotransmitter acetylcholine (Zeisel and Blusztajn, 1994). It is not yet known how choline is able to mitigate alcohol-related learning deficits; however, prenatal choline supplementation in typically developing rats (not alcohol-exposed) modulates signaling of hippocampal cholinergic neurons (Blusztajn et al., 1998), an effect that is observed months after choline supplementation is complete (Meck and Williams, 2003). Given that the cholinergic system modulates memory and attentional processes (Ridley et al., 1989; Furey et al., 2008), it is possible that perinatal choline supplementation may be altering cholinergic functioning in subjects exposed to alcohol during development.

Developmental alcohol exposure can cause deficits in hippocampal-based learning and memory, as well as long-lasting changes in hippocampal muscarinic acetylcholine receptor (mAChR) density. There have been several studies that have used subtype-unspecific ligands to examine muscarinic receptor density in animal models of perinatal alcohol exposure. For example, prenatal alcohol exposure leads to an increase in hippocampal muscarinic receptors in both young (PD 4) and adolescent (PD 30) rats (Nio et al., 1991). Similarly, alcohol exposure during the 3rd trimester equivalent brain growth spurt increases the density of hippocampal muscarinic receptors observed during adulthood (Kelly et al., 1989). Similar results were found in 21-day-old rats exposed to ethanol during the combined prenatal and neonatal periods (Carneiro et al., 2005). Increases in muscarinic receptors may reflect compensatory reactions to acute and long-term ethanol-induced reductions in acetylcholine levels following prenatal alcohol exposure (Rawat, 1977). In contrast, perinatal choline supplementation in otherwise typically developing rats increases acetylcholine (ACh) release, while reducing choline turnover through choline transporters (Blusztajn et al., 1998). The present study examined whether perinatal choline supplementation influences hippocampal cholinergic systems in subjects exposed to alcohol during development by measuring the densities of several subtypes of muscarinic receptors. Rats were exposed to alcohol during the 3rd trimester equivalent brain growth spurt, which occurs postnatally in the rodent, and treated with choline during and after alcohol exposure.

MATERIALS AND METHODS

Subjects were 53 male offspring of Sprague-Dawley rats bred at the San Diego State University Animal Care Facilities. Mothers were maintained in a temperature- and humidity controlled environment with food (LabDiet 5001; Richmond IN, which contains 2.25 g/kg choline chloride) and water available *ad lib*. Multiparous dams were housed overnight with males. The presence of a sperm plug on the following morning designated gestational day 0. On the day following birth, litters were culled to 7 pups, retaining no fewer than 4 male subjects.

Subjects were exposed to alcohol on postnatal days (PD) 4-9, a developmental period comparable to the 3rd trimester in humans, which is characterized by rapid CNS development (Dobbing & Sands, 1979). A male from each litter was randomly assigned to one of four treatment groups in a 2 (Ethanol, Control) $\times$ 2 (Choline, Vehicle) design. On PD 4-9, ethanol groups received intragastric intubations of a nutritionally balanced milk diet containing ethanol (5.25 g/k/day; 11.9% v/v) divided in 2 daily feedings, 2 hours apart. Since ethanol-exposed subjects suckle less when intoxicated, they were given milk supplements via intubation 2 and 4 hours after ethanol treatment each day. Controls subjects received sham intubations to control for any stress incurred by the intubation procedure. Between intubations, subjects were returned to the dam and monitored throughout the day.

Half of the subjects in each group received 100 mg/kg/day choline chloride via daily subcutaneous injections from PD 4 – 30, a period of development during which choline effectively attenuates learning deficits associated with perinatal alcohol exposure (Thomas et al., 2004). The other half of the subjects received saline vehicle injections. From PD 4-7, individual subjects were identified by marking the tail with a black marker and on PD 8, subjects were coded via injections of India ink into the paws. This coding system allowed investigators to identify each individual pup while remaining blind to treatment condition during behavioral testing and histological analysis.

Blood Alcohol Level

On PD 6, twenty microliters of blood was drawn from the tail of each pup and collected into micropipettes 1.5 hr after the last daily alcohol intubation to determine peak blood alcohol concentrations. Blood samples were analyzed with an Analox AM1 alcohol analyzer, which uses an enzymatic oxygen-rate analyzing method across a Clark-type amperometric oxygen electrode (Analox Instruments Ltd, UK).

Open Field Activity

Hyperactivity is a common behavioral outcome of developmental alcohol exposure (Coles et al., 1997) and may be related to hippocampal dysfunction (Gaspar and Duncan, 2009). On PD 30-33, activity level was monitored in Plexiglas open fields (40 $\times$ 40 $\times$ 30.5 cm) equipped with optical beams that recorded each subject's movements (Hamilton-Kinder, LLC). The monitors and cages were located in dark enclosed chambers, equipped with fans to provide airflow. Computer-generated white noise served to mask any outside noises. Subjects were placed in the dark testing room (lit by a red-light) 30 minutes prior to testing to allow for acclimation. Before testing each subject, the chambers were washed with a 20% ethanol/water solution to remove odor cues. Each subject was then placed in the center of the open field chamber, and interruptions of the infrared beams are recorded electronically. A variety of activity measures were automatically collected every 5 minutes during the daily 60-min test, which was conducted during the animal's dark cycle (1800 – 2300). Behavioral measures such as total distance traveled, time spent in the center of the chamber, and rearing were examined.

Receptor Binding Assays

Animals were euthanized by decapitation on PD 35 for tissue collection. Brains were extracted quickly, frozen in 2-methylbutene (cooled with dry ice), and wrapped in air-tight packages for storage at -80°C until use. The brain tissue was cut in twenty-micron coronal sections at -20°C and thaw-mounted onto cold, positively charged, glass slides, and adjacent sections were collected for determination of total and nonspecific ligand binding. For each receptor assay, six coronal sections were taken at 160 μm intervals between 2800 μm – 3800 μm post-bregma for both total and non-specific binding, throughout the entire dorsal hippocampus. The slides were desiccated at 4°C for 2 h and stored at -20°C for no

more than 2 days prior to processing in radioligand binding assays. Muscarinic (M_1 & $M_{2/4}$) receptors were then measured.

Optimal binding conditions for each radioligand were determined with the protocol described by Smith, Gallagher, & Leslie, and are shown in Table 1 (Smith et al., 1995). Radioligands were purchased from New England Nuclear (Boston, MA). Brain slices were defrosted for 1 h at room temperature. The sections were washed to remove endogenous ligand, and were then incubated with the respective radioligand in the presence and absence of an unlabeled competitive ligand to define total and nonspecific binding. The sections were then washed to remove unbound radioligands from the slides, and were dried for a minimum of 30 min. Tissue sections and tritium standards (Amersham, Arlington Heights, IL) were co-exposed to radiation-sensitive film (Kodak BioMax Film) in light-tight cassettes. Following a predetermined exposure period, the film was developed for quantitative analysis.

Muscarinic M_1 subtype receptors were labeled with the radioligand [3H]pirenzepine (10nM; ~120Ci/mM) and muscarinic $M_{2/4}$ subtype receptors were labeled with the radioligand [3H]AF-DX 384 (7nM; ~120Ci/mM) (Birdsall et al., 2010). Specific binding was determined by the difference between total binding, and binding in the presence of atropine (1 μ M). Autoradiographic images were quantified using a video-based computerized image analysis system (MCID, Imaging Research Inc., St. Catharine, Ontario, Canada). Calibration curves of optical density versus radioactivity were generated using tritium-labeled microscale standards (Amersham Biosciences). Optical densities of the dorsal hippocampus were measured and corresponding radioligand binding values were determined by interpolation from the standard curve.

Data analysis

Data were analyzed with ANOVAs using SPSS software. Body weights, open field activity data, and ligand binding densities were analyzed using a general linear model with repeated measures. Ethanol exposure (EtOH, Sham) and choline treatment (Choline, Vehicle) served as between-subject factors. Activity level was analyzed using day and 5-min bins as repeated measures. Body weight was analyzed using day as a repeated measure. Muscarinic receptor density was analyzed in the dorsal hippocampus with six slices (from anterior to posterior) as repeated measures.

RESULTS

Body weight

Body weights were measured daily from PD 4-30. Body weights were analyzed separately for the ethanol treatment period (PD 4-10) and later development (PD 11-30). From PD 4-10, all groups gained weight across days, producing a main effect of day [$F(6, 312) = 1605, p < 0.05$] (see Figure 1A). There was a significant day by ethanol interaction [$F(6, 312) = 61.1, p < 0.05$] and main effect of ethanol [$F(1, 52) = 30.6, p < 0.05$]. Although there were no significant body weight differences between treatment groups on the first intubation day (PD 4), ethanol-exposed subjects gained less weight than control subjects across days, differing significantly beginning on PD 5. When data from PD 11-30 (Figure 1) were analyzed, there was still an effect of ethanol [$F(1, 52) = 7.3, p < 0.05$] and day [$F(19, 988) = 47464, P < 0.05$], as ethanol-exposed subjects continued to weigh less than sham controls. However, when data were analyzed on PD 30, there were no longer effects of ethanol, indicating that the ethanol-exposed subjects caught up in body weight. Importantly, choline supplementation had no effects on body weight at any time.

Blood alcohol content

Blood alcohol content (BAC) was not significantly different between treatment groups receiving ethanol [$F(1,25) = 0.004$, $p = 0.95$]. In fact, the mean BACs were remarkably similar; ethanol-exposed subjects receiving choline had an average BAC of 334.3 ± 6.9 mg/dl whereas the average BAC for the ethanol-exposed group receiving saline vehicle was 333.5 ± 10.9 mg/dl.

Open field activity

Total distance traveled in the open field chamber is shown in Figure 2. All groups reduced activity over days and bins producing a main effect of day [$F(3, 150) = 33.0$, $P < 0.05$], bin [$F(11, 550) = 498.6$, $P < .05$], and an interaction of day and bin [$F(33, 1650) = 11.2$, $P < .05$]. There were also interactions of day by ethanol [$F(3, 150) = 3.1$, $P < .05$] and bin by ethanol [$F(11, 550) = 3.9$, $P < .05$], as the ethanol-exposed subjects were more active compared to controls during the first days of testing as well as at the beginning and end of the sessions. Although the ethanol by choline interaction failed to reach statistical significance in the overall analysis, when data during the first 10 min and last 20 min of each session were analyzed during the first two days, there was a significant ethanol by choline interaction [$F(1,50) = 4.9$, $p < .05$]. Follow-up comparisons confirmed that ethanol-exposed subjects treated with choline were significantly less active compared to ethanol-exposed subjects treated with vehicle. In fact, throughout testing, activity levels of the ethanol-exposed subjects treated with choline were not statistically different than that of either control group.

Cholinergic Receptor Density

Some tissue sections did not process correctly, thus the N varies between the open field test and the binding assays. Alcohol exposure during the 3rd trimester equivalent significantly reduced the density of M_1 receptors [$F(1,29) = 6.2$, $p < 0.05$] (see Figure 3, Panel A). There was no effect of choline on M_1 receptor density in the hippocampus. In contrast, there was a significant interaction of ethanol and choline in the density of $M_{2/4}$ receptors [$F(1,32) = 6.2$; $p < .05$]. The hippocampus of ethanol-exposed pups had an elevated density of $M_{2/4}$ receptors (see Figure 3B), an effect that was significantly attenuated by treatment of choline. In fact, $M_{2/4}$ receptor density did not differ significantly between the ethanol-exposed subjects treated with choline and either control group.

When measuring the ratio of M_1 to $M_{2/4}$ receptor subtypes, there was a significant interaction of ethanol and choline [$F(1,24) = 4.8$; $p < .05$]. The hippocampus of ethanol-exposed pups had a significantly lower ratio of M_1 to $M_{2/4}$ receptors compared to all groups, an effect that was significantly attenuated by treatment of choline (see Figure 4, panel B). Interestingly, the ratio of M_1 to $M_{2/4}$ densities did not differ significantly between the ethanol-exposed group treated with choline and controls.

DISCUSSION

The present study suggests that choline supplementation may attenuate some of the adverse effects of developmental alcohol exposure by altering hippocampal cholinergic systems. Although perinatal choline supplementation did not influence ethanol-related reductions in M_1 receptor density, it did mitigate the ethanol-related increases in $M_{2/4}$ receptor density. These data suggest that although choline supplementation does not affect all alcohol-related changes in hippocampal cholinergic systems, it does influence some. Importantly, choline did not influence blood alcohol level, indicating that these effects were not simply due to changes in alcohol metabolism. This is the first demonstration that choline supplementation alters neural structure in subjects exposed to alcohol during development.

Consistent with previous studies, developmental alcohol exposure altered development of the hippocampal cholinergic system. Studies that examined the effects of early alcohol exposure on cholinergic systems utilized the muscarinic antagonist quinuclidinyl benzilate (QNB) for their binding assays, which is non-selective for the various muscarinic receptor subtypes. Using QNB, studies have reported that developmental alcohol exposure increases the number of hippocampal muscarinic receptors (Kelly et al., 1989; Nio et al., 1991; Carneiro et al., 2005). The present study used ligands with a higher degree of subtype specificity, pirenzepine for M_1 and AF-DX 384 for $M_{2/4}$, to examine changes in receptor subtypes and found that alcohol reduced M_1 receptors and increased $M_{2/4}$ receptors. The average combined M_1 and $M_{2/4}$ density of the ethanol-exposed group in this study was greater than the control group (see Figure 4A), which is consistent with previous studies; however, using subtype-specific ligands we find this effect is driven primarily by increases in inhibitory $M_{2/4}$ muscarinic receptors.

Perinatal choline supplementation also affects long-term hippocampal cholinergic functioning. In typically developing rats, combined pre- and neonatal choline supplementation results in a long-term decrease in hippocampal choline acetyltransferase levels coupled with an increase in muscarinic receptors (as measured using the subtype unselective QNB ligand) (Meck et al., 1989). The present study administered choline exclusively during postnatal development and receptors were examined within a few days of supplementation. Nevertheless, the results are consistent with previous findings. In the present study, choline-supplemented controls did have more muscarinic receptors overall (Figure 4A), although the ratio of M_1 : $M_{2/4}$ receptor subtypes did not differ between controls that received choline or vehicle. Thus, both choline supplementation and developmental alcohol exposure led to an overall increase in the density of muscarinic receptors; however, the ratio of receptor subtypes varied with treatment, with the ethanol-exposed subjects not treated with choline having the lowest ratio of M_1 to $M_{2/4}$ receptors compared to the other groups.

The ratio of M_1 to $M_{2/4}$ receptors could have important downstream influences on gene expression and memory. Muscarinic receptors are linked to G-proteins where M_1 activation increases cAMP and $M_{2/4}$ receptor activation decreases cAMP. Accumulation of cAMP activates the transcription factor CREB (cAMP Response Element Binding protein), which is essential for memory formation (Davis and Squire, 1984), and has been shown to increase expression of brain derived neurotrophic factor (BDNF) (Tao et al., 1998; Zha et al., 2001). CREB and BDNF have a well-documented role in neuronal plasticity and long-term memory formation in the brain (Alonso et al., 2002; Brightwell et al., 2005; Chen et al., 2010) and, in fact, prenatal choline supplementation in typically developing rodents increases CREB phosphorylation (Mellot et al., 2004). We find that the density of M_1 receptors is decreased in both groups of ethanol-exposed subjects, however, the density of $M_{2/4}$ receptors is elevated only in the ethanol group that didn't receive choline treatment. Thus the hippocampus of ethanol-exposed subjects may have less activation of cAMP and downstream signals. This could be one mechanism by which choline supplementation mitigates alcohol-related learning deficits.

Consistent with changes in hippocampal cholinergic development, this study confirms that developmental ethanol exposure can result in behavioral alterations which can be mitigated by choline supplementation early in development. Specifically, ethanol exposure during the third trimester-equivalent resulted in hyperactivity in the open field, an effect attenuated by choline treatment. This is consistent with previous reports that choline supplementation during the perinatal period reduces the severity of open field overactivity (Thomas et al., 2004), spatial working memory deficits (Thomas et al., 2010), trace eyeblink conditioning deficits (Thomas and Tran, 2010) and trace fear conditioning deficits (Wagner and Hunt,

2006) associated with developmental alcohol exposure, all of which may rely on the functional integrity of the hippocampus and related forebrain structures. Previous experiments have demonstrated a link between cholinergic functioning and performance on hippocampal-dependent tasks, including open field activity. In one study muscarinic M₁ knockout mice displayed a marked increase in ambulatory activity (Miyakawa et al., 2001). This parallels our findings in that the ethanol-exposed group had the least amount of M₁ expression and the highest activity levels.

This behavioral test was chosen rather than other hippocampally based learning tasks so that we could confirm that ethanol and choline were having functional effects without exposing subjects to a behavioral task known to directly modulate cholinergic markers (van der Zee and Luiten, 1999). In contrast, other hippocampal-related learning tasks themselves have been shown to modulate muscarinic receptor expression. For instance, rodents trained on both spatial learning and trace eyeblink tasks display increases in mAChR (van der Zee and Luiten, 1999), and levels of mAChR density may reflect the degree of training on the hippocampal based task with behavioral performance (Beldhuis et al., 1992; van der Zee and Luiten, 1999). Although it is possible that exposure to the open field could have influenced mAChR density, it does not discount that the expression differs among developmental alcohol-exposed subjects and that choline modifies alcohol's effects on cholinergic receptors.

It is important to note that levels of receptor expression may change over time. In the present study, receptor densities were assessed 25 days after alcohol exposure and 5 days after choline supplementation was complete. It will be important to determine if the effects of choline supplementation on cholinergic systems in the alcohol-exposed subjects are long-lasting. Previous studies with typically developing rats have reported that perinatal choline supplementation leads to long-lasting changes in cholinergic functioning, up to PD 75 (Meck et al., 1989). Choline availability during a critical perinatal period has been proposed to cause metabolic imprinting and relatively permanent alterations in cholinergic neuron signaling (Meck and Williams, 2003). However, the effects of choline supplementation specifically administered during the first few postnatal weeks have not been examined. It will also be important to investigate whether similar changes are seen in other brain regions and whether other components, such as nicotinic AChR and choline transporters, are also altered by early alcohol and choline treatment.

One final caveat is that ethanol-exposed subjects in the present study lagged in growth compared to controls. Importantly, we have replicated the effects of both developmental alcohol exposure and choline supplementation on behavior (including open field activity (Thomas, et al. 2004)) using an artificial rearing procedure that controls for nutrition and maternal care. Moreover, using an artificial rearing procedure for alcohol administration from PD 4-9, Kelly et al (1989) reported an increase in muscarinic receptors (as measured with QNB), which is consistent with our findings. Finally, choline supplementation did not influence body weights, so choline modification of alcohol-related changes in muscarinic receptors is unlikely to be related to growth. That being said, it is not yet known whether alcohol significantly influences choline levels, either by altering intake or by affecting choline absorption or utilization, and it is possible that choline may compensate for an alcohol-induced deficiency in the present study. It is also not known if alcohol interacts with intubation stress in unique ways that are affected by choline.

The present study illustrates that choline can alter hippocampal cholinergic systems in alcohol-exposed subjects. However, it is important to note that choline has many actions outside its role as a precursor to the neurotransmitter acetylcholine. Choline acts as a methyl donor, and can influence methylation patterns, including DNA methylation (Niculescu et al.,

2006). Choline can also influence neuronal phospholipid profiles and is implicated in other signaling pathways (Zeisel and Blusztajn, 1994). Developmental alcohol exposure also affects these targets. Thus, although the present study suggests that choline modified hippocampal cholinergic functioning, this is certainly not likely to be the only site of choline's action.

As we explore possible therapeutics for reducing alcohol's teratogenic effects, elucidation of the mechanisms by which these experimental therapeutics are effective may lead to development of additional, more targeted interventions, and to the identification of treatment parameters that will be most effective. For example, choline supplementation can reduce the severity of alcohol's teratogenic effects when administered either during the prenatal period (Thomas et al., 2010) or postnatally, after alcohol exposure (Ryan et al., 2008). It is not clear if choline supplementation would have similar effects on cholinergic activity at each of these development periods, or how the effects of choline may depend on the dose, developmental timing and duration of treatment. Nevertheless, the present study provides the first demonstration that choline alters the hippocampal cholinergic system among subjects exposed to alcohol during development, providing one possible neural substrate for the behavioral mitigation of ethanol's effects afforded by choline. Elucidation of choline's effects on the alcohol-exposed brain may provide essential information on how to utilize choline as a treatment for FASD in the most effective way.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1**Radioligand Binding Procedure**

Radioligand	Competing Ligand	Pre-Incubation	Incubation	Wash
[³ H]Pirenzepine 10 nM	1 μ M Atropine	Krebs-Hepes pH 7.4 10 min 22°C	Krebs-Hepes pH 7.4 60 min 22°C	Krebs-Hepes pH 7.4 2 $\times$ 2 min 1 dip dH ₂ O 4°C
[³ H]AF-DX 384 7 nM	1 μ M Atropine	Krebs-Hepes pH 7.4 10 min 22°C	Krebs-Hepes pH 7.4 120 min 22°C	Krebs-Hepes pH 7.4 2 $\times$ 2 min 1 dip dH ₂ O 4°C

* Krebs-Hepes: 0.1 M NaCl, 2.0 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 5.0 mM HEPES, 5.5 mM glucose