CONNEXIN36 EXPRESSION IN HYPOGLOSSAL MOTONEURONS OF NORMAL AND NICOTINE TREATED, NEONATAL RATS

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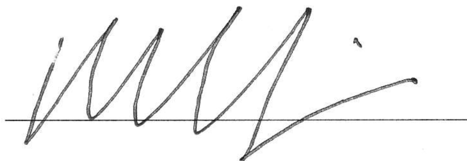

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Connexin36 Expression in Hypoglossal Motoneurons of Normal and Nicotine Treated, Neonatal Rats

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Abstract

Problems in respiration occur that can lead to ineffective breathing and, eventually, death. A lot of these problems occur after nicotine exposure during fetal development. Motoneurons of the hypoglossal are responsible for regulating respiration. In normal development, motoneurons in the hypoglossal nucleus are highly interconnected through the presence of gap junctions until birth when their presence begins to disappear. Problems may occur if the high expression of gap junctions, specifically connexin36 (Cx36) remains after birth. An antibody directed against Cx36 (Zymed 51-6200) was used to identify Cx36 expression in the brain stem tissue of rat neonates of different ages. The neonates were either exposed to nicotine during fetal development or not in order to produce a comparison. Of the samples produced, eleven had successful staining. This was not enough to determine whether or not there is a difference in connextion36 expression between rats that were treated with nicotine or not. Although no conclusions can be drawn, it has identified several different potential areas of study, including looking into different connexins or using a different antibody.

Introduction

1. Background

The coordination of the lungs, diaphragm, and upper airways is present from birth to death in most animals. Autonomic and voluntary signals from motoneurons in the central nervous system regulate the movement of the diaphragm that allows for ventilation and exhalation of the lungs (Feldman and Shao 2009). Specifically, a region in the brain, called the pre- Bötzinger has been found to be responsible for generating respiratory frequency and rhythm generation. Although the exact mechanism behind this process is still unclear, the process normally allows for effective, involuntary breathing in most animals. However, problems can arise during development that cause this system to malfunction (Blessing, 2004; Pattinson et al., 2009; Schwarzacher, Rüb, and Deller 2011).

Problems in respiration occur that can lead to ineffective breathing and, eventually, death. Most of these diseases, including sudden infant death syndrome (SIDS), present themselves at birth or during the first few weeks of life because a working respiratory system is necessary for survival. SIDS is caused by the sudden cessation of breathing, referred to as apnea. However, the damage of SIDS is caused before birth, during development. Tobacco smoke exposure has been named the number one risk factor for developing SIDS and it has been estimated that tobacco exposure is responsible for about one-third of all SIDS deaths (Anderson et al. 2005; Mitchell and Milerad 2006; Fregosi and Pilarski 2008). Specific defects seen in nicotine-exposed neonatal animals include lowered ability to detect and respond to hypoxia (Hafstrom et al. 2000; Lewis and Bosque 1995; Bamford and Carroll 1999; Bamford et al. 1996; Fewell et al. 2001a,b; Froen et al. 2002; Hafstrom et al. 2005; St-John and Leiter 1999),decreased ability to auto-resuscitate after severe hypoxic exposure (Fewell and Smith 1998; Froen et al. 2000), reduced ventilatory

output (Huang et al. 2004; St-John and Leiter 1999), higher rate and duration of apnea (Fewell et al. 2001a; Huang et al. 2004; Froen et al. 2002), and irregularity in breathing pattern (Fewell et al. 2001a; Hafstrom et al. 2002; Huang et al. 2004). Therefore, it tobacco exposure could possibly facilitate or, even cause, SIDS and other developmental breathing disorders.

Through the study and treatment of problems that arise during development, the brain's regulation of the respiratory system has become clearer. As stated previously, the Pre-Bötzinger complex of the medullary rhythm-generating network is essential for respiratory rhythm in mammals (Smith et al., 1991). It is composed of a cluster of interneurons, which drive the respiratory pattern. A study by Feldman and McKay 2008 found that silencing neurons in the pre-Bötzinger complex lead to sleep disordered breathing in rodents. In particular, an area called the hypoglossal nucleus, which runs the length of the medulla and is composed of the motoneurons critical for respiratory development and regulation (Feldman and Del Negro 2006). Problems with this area in particular could explain developmental problems in respiration.

In normal development, motoneurons in the hypoglossal nucleus are highly interconnected through the presence of gap junctions (Cifra et al. 2009). This allows for the direct exchange of information and activation signals from one motoneuron to another (Cifra et al. 2009). This is important because, while in the womb, the cells that regulate respiration need to be able to active with the smallest of signals (Rekling, Shao, and Feldman 2000). In order to assure that this happens, gap junctions allow for the signal to travel quickly between cells to activate more before the signal fades. However, with birth, the presence of these gap junctions begins to disappear because it order to successfully regulate gas exchange, all the cells of the hypoglossal cannot fire at once (Cottrell and Burt 2005). If the cells were to fire all at the same time, it could cause respiratory spasms and interrupt normal breathing (Cottrell and Burt 2005).

Therefore, the presence of gap junctions in the hypoglossal normally disappear within the first couple weeks of life (Rekling, Shao, and Feldman 2000). However, this is not always the case. It has been predicted that the presence of gap junctions long into postnatal development may be associated with respiratory diseases (Greer and Funk 2005). Unfortunately, not much is known about the correlation or relationship between gap junction presence and disease occurrence. One possible cause of irregular gap junction development could be prenatal nicotine exposure.

It has been found that the pre-exposure to certain toxins, such as nicotine, during gestation, can cause an interruption in normal development of the hypoglossal nucleus (Fregosi and Pilarski 2008). It is known that nicotine interacts with prime nicotinic acetylcholine receptors (nAChRs). From there it has been suggested that it causes an up-regulation of nAChRs on pre-synaptic neurons to the preBötzinger complex region (Barazangi and Role 2001; Dajas-Bailador and Wonnacott 2004; Hsieh et al. 2002; Vizi and Lendvai 1999; Fregosi and Pilarski 2008). From there, Fregosi and Pilarski 2008 predict that constant stimulation of these receptors may lead to overuse and desensitization. The reduction in activation causes an up-regulation of GABA and glutamate receptors on the post-synaptic neuron, which would lead to an increased sensitivity. This is one theory of how nicotine may cause respiratory problems, but there is still a lot more research that must be done. Nicotine may affect different areas that have not been identified yet.

Since it has been suggested that the negative effects of nicotine-exposure may be caused by an increase in the firing rate of certain neurons, it is reasonable to wonder if it may have an effect on gap junctions as well. Perhaps the effects induced by nicotine exposure may be heightened or partly caused by irregular gap junction presence, which would allow for all cells in the area to be affected when one is activated. Therefore, all cells would experience the effects of

nicotine. This would help explain why nicotine-exposure seems to affect both inhibitory and excitatory neurotransmission in the brain (Fregosi and Pilarski 2008). Yet, no one has looked into the developmental changes of specific gap junctions due to prenatal nicotine-exposure. Therefore, a closer look is necessary to completely exclude or support the possibility.

Gap Junctions

In order to understand the role of gap junctions, the definition of a gap junction must be understood first. It has been found that gap junctions are intercellular channels that create a metabolic and electrical coupling between neighboring cells by allowing a direct exchange of small molecules and ions (Solomon and Dean 2002). Recent effort has been made to establish the functional role of gap junctions. So far, it has been determined that, gap junctions create metabolic cooperation and long range signaling through the passage of ions, small metabolites, and second messengers (Parenti et al. 2000). However, in addition to the previously mentioned functions, gap junctions play an important role in development. Gap junctions are important in CNS development and are a part of corticogenesis, cell proliferation, cell migration, coordination of neuronal differentiation, and neuronal circuit formation (Dermietzel et al. 1989; Guthrie and Gilula 1989; Kandler and Katz 1995; Nadarajah et al. 1997; Solomon and Dean 2002).

Although there are several different types of gap junction throughout the body, they all share the same general structure. In order to make a gap junction, two hemichannels (connexons) of two adjoining cells align to form a pore. These connexons are composed of a hexameric arrangement of structural proteins, called connexins (Solomon and Dean 2002). Fifteen different types of connexins have been identified in the rodent and can be found throughout the body including the central nervous system (Solomon and Dean 2002). Out of the fifteen connexins that have been identified, so far, only nine have been reported to be expressed in the adult and/or

developing CNS. Of these nine, the most abundant include Cx26, Cx32, Cx43, Cx30, and Cx36 (Solomon and Dean 2002). Further research has determined that Cx26, Cx32, and Cx36 are found in neurons. While Cx32 has also been found in oligodendrocytes and Cx26 has also been found in atrocytes, Cx36 predominantly found in neurons of the CNS (Solomon and Dean 2002). What makes Cx36 even more interesting is it appears in high concentrations throughout the motor pathway, olfactory pathway, hippocampus, and areas responsible for respiratory rhythm (Parenti et al. 2000). This combined evidence suggests that the identification of developmental differences in Cx36 expression in the hypoglossal motoneurons may be extremely important. That is why Cx36 was the focus of this paper.

As stated before, in normal development, connexin presence disappears exponentials with age in the hypoglossal nucleus (Condorelli et al. 2000). General developmental patterns of Cx36 follow this observation. Its concentration drastically increases during early postnatal development, but then declines to adult levels (Sohl et al. 1998; Belluardo et al. 2000). From Condorelli et. al 2000, the general presence of Cx36 concentration throughout development has be determined. There is a peak in concentration around day 7 and day 16. They, also, appear in high concentrations across the developing brain during development that is not seen in adulthood. The concentration of Cx36 disappears around day 20 in rodents. Finally, although scattered throughout the brain during early development, Cx36 begins to disappear deep to superficial throughout the cortex. Currently, knowledge about the exact developmental pattern across the hypoglossal motoneurons is needed.

Predictions

In this paper, the developmental pattern of Cx36 in the hypoglossal motoneurons is explored through the use of histochemistry. By mapping out the developmental patterns of Cx36

from post-natal day 3 to day 20, hopefully further knowledge about how the roles of gap junctions affect respiratory development may be uncovered. Both normal and nicotine pre-treated, neonatal rats will be used. Animals that are exposed to nicotine during fetal development develop a wide range of problems after birth, including respiratory problems (Fregosi and Pilarski 2008). However, it is unknown whether or not a difference in gap junction expression is to blame. From the data, it is hoped that the normal developmental pattern of Cx36 expression may be determined based on the evidence collected from animals that were not exposed to nicotine. It is predicted that the nicotine-exposed animals will have a different Cx36 expression, specifically a higher concentration of Cx36 for a longer than normal period of time. This presence would allow for increased cell communication possibly leading to respiratory failure.

Methods

Animals

Both nicotine-exposed and not exposed neonatal rats were used between the ages of 0P and 20P. Nicotine-exposed neonates were created by implanting an osmotic minipump into the pregnant dam on the fourth or fifth day of gestation. The pump delivered nicotine bitartrate at a level of 6 mg/(kg day). Indicating that there was about 24 ng/ml in neonates and 18 ng/ml in pregnant dams. This can be directly compared to the amount of nicotine that would be in the system of a pregnant moderate smoker because higher doses are needed in rats to achieve the same effects seen in humans (Lichtensteiger et al. 1988; Slotkin 1998). The pump remained in the female rat after birth in order to continue the nicotine exposure through postnatal suckling. In order to directly compare normal development versus nictotine-exposed, the same age range was used between the two sets of animals.

Histochemistry

In order to identify Cx36 expression, sections of the hypoglossal were mounted in order on electrostatic glass slides. The slides were blocked for one hour with 0.25% Bovine Serum Albumin (BSA) and 0.1% Triton X-100 in 0.1 M Tris buffer (pH 7.4). Next, the sections were incubated overnight with an antibody directed against Cx36 (Zymed 51-6200). The primary antibody dilution was 1:8000.

The sections were, then, rinsed and incubated appropriate biotinylated secondary antibody solution (dilution of 1:100) for two to four hours. The sections were then washed and incubated in avidin-biotin-HRP complex (Vector Labs Elite kit) made in 0.1 M Tris buffer. For visualization, the sections were incubated in a 100 mL solution containing 50 mg of the chromagen 3,3-diaminobenizidine, 40 mg ammonium chloride, 0.3 mg glucose oxidase (Sigma),

and 200 mg β-D+-glucose. To check the rate of staining, incubation was examined occasionally with a light microscope. Once desired staining was obtained, the process was stopped by rinsing the slides in 0.1 M Tris buffer three times, dehydrated, secured with cover slips, and examined with light microscope.

Data Analysis

Using the slides of a predetermined section of the hypoglossal, a high quality picture of each section of brainstem tissue was obtained. The photos were uploaded onto a computer program that calculated with number of Cx36 present. A comparison between nicotine-exposed and saline-exposed animals was conducted by examining the slices from neonates of the same age.

Results

Figure 1:

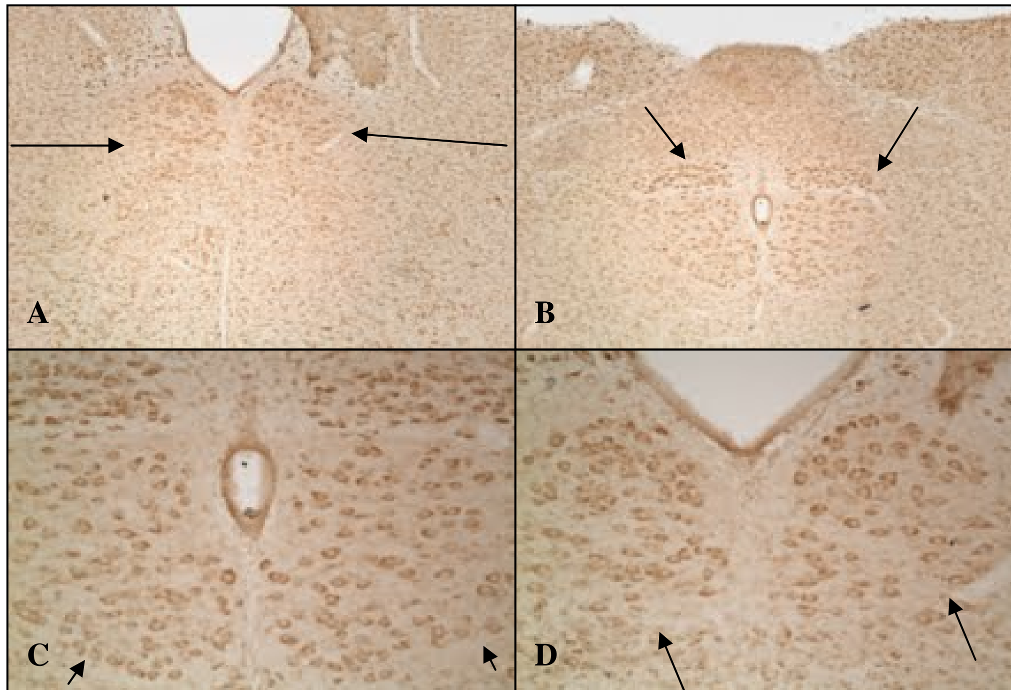

Figure 1 shows an example of staining obtained from saline P15 rat tissue. The darker regions indicate where connexin36 is located. The arrows point to the hypoglossal motor nucleus. A and B were taken using 4x magnification, while C and D were obtained using a 10x magnification.

Figure 2:

Date Sliced	Concentration of Antibody	Age of Specimen	Nicotine Exposure	Successful Staining?	Batch of Antibody
11/24/2010	1 to 5000 (original titration)	15	N	Y	Original
11/30/2010	1 to 5000	12	Y	Y	Original
11/30/2010	1 to 5000	12	N	Y	Original
1/11/2011	1 to 5000	12	Y	Y	Original
1/11/2011	1 to 5000	12	N	Y	Original
1/21/2011	1 to 5000	3	N	Y	Original
2/3/2011	1 to 5000	9	Y	N	Original
2/3/2011	1 to 5000	9	N	N	Original
2/20/2011	1 to 5000	6	Y	N	Original

2/20/2011	1 to 5000	6	N	N	Original
2/22/2011	1 to 5000	6	Y	Y	Original
2/22/2011	1 to 5000	6	N	Y	Original
2/24/2011	1 to 5000	9	Y	N	Original
2/24/2011	1 to 5000	9	N	N	Original
3/3/2011	1 to 5000	3	Y	N	Original
3/3/2011	1 to 5000	3	N	N	Original
3/9/2011	1 to 5000	9	Y and N (on same slide)	N	Original
3/31/2011	1 to 1000 (titration)	2 and 16 (on same slide)	N	Y	New
4/7/2011	1 to 1000	9	Y and N (on same slide)	Y	New
4/13/2011	1 to 1000	3	Y and N (on same slide)	Y	New
4/15/2011	1 to 1000	6	Y and N (on same slide)	Y	New

Figure 2 shows which samples were run and whether or not they yielded results for the connexin36 antibody.

Only eight successful staining samples were obtained from ages P3, P6, P12, and P15 using the antibody concentration of 1:5000, which was the original concentration that the antibody was titrated at on November 24th, 2010. Figure 1 illustrates successful staining indicating where connexin36 in the hypoglossal nucleus was located. Figure 2 shows which samples were run and whether or not successful staining occurred. The number of unsuccessful staining samples began to increase half-way during the experiment.

On January 11th, 2011 problems with staining began to occur. The antibody stopped producing results for younger tissue samples (>P12). It appeared as though the antibody was no longer reacting with the tissue samples. In order to try to correct this situation and determine if it was a problem with concentration, the antibody was titrated using both young (P2) and older (P16) tissue on March 31st, 2011. A new concentration of antibody was determined at 1:1000 and yielded more results with successful staining. As seen in Figure 2, three more successful staining

samples have been acquired since then at ages of P9, P3, and P6. In addition, a new bottle of antibody was ordered and began to be used on March 31st, 2011.

Discussion

According to previous research, it has been determined that connexin36 expression for neonatal rodents decreases with age with a peak in concentration around P7 (Condorelli et. al 2000). The antibody for connexin36 stopped producing results in younger tissue samples (>P6) over the course of the experiment. The fact that previous research predicted a higher concentration at a younger age, led to the conclusion that something may be going wrong. Some of the possible issues that could have caused the problems include over fixation of the tissue samples, the wrong antibody to explore hippocampus, the wrong concentration of antibody, the antibody expired over the course of the experiment, and the possibility that the antibody does not recognize rat tissue.

Although the schedule as written in the procedure was followed as closely as possible, there were a couple of times where the samples were left too long in certain solutions. The timing for the procedure was determined based on what had worked for other antibodies for rat tissue from similar age groups. There was some issue in removing the tissue samples out of primary according to the time schedule. This could have contributed to the antibody problem because over exposure could have affected the resulting staining processes. This cannot be the main reason because the issue occurred even in samples where the protocol timing was followed exactly as written.

Connexin36 was selected as the gap junction protein to explore because it is located in the central nervous system and was present in neurons as opposed to oligodendrocytes or atrocytes (Solomon and Dean 2002). This may be the wrong connexin to obtain the information required. Although previous research has found connexin36 present in the hypoglossal brain region of neonatal rats (Sohl et al. 1998; Belluardo et al. 2000), the relationship between

motoneurons, nicotine, and connexin36 has not been studied. There are five known connexins located in the central nervous system. This issue determining connexin36 concentration for some of the tissue samples may be due to looking into the wrong connexin. Research into the other four connexins will determine if connexin36 was a good choice for this project. This does not explain why it was difficult to get successful staining for younger samples because it differs from what previous research determined (Condorelli et al. 2000).

After the antibody was titrated, the issue seemed to disappear. It could be that it was caused by the wrong antibody concentration. Not many samples have been completed since this issue, so the problem could recur. This does not explain why successful staining was achieved in the beginning of the experiment.

A new bottle of antibody was purchased and began use on March 31st, 2011. The problem with staining may have resulted from the original antibody going bad. The original antibody was allocated and placed in the freezer in November 2010. It may have just gone bad over time or have been defective because the new antibody seems to be producing successful staining.

The antibody may not recognize rat tissue. The antibody was made for mice tissue. Although the Zymed website indicates that the antibody should work for rat tissue, no previous papers have been published using the antibody on rat tissue. The antibody may not work as well as it should when applied to rat tissue. This could explain the problems obtaining results from younger tissue samples.

It has been determined that no conclusion can be drawn from the results. More results are necessary to determine if connexin36 plays a role in respiration and what effect nicotine may have on it. In order to get more results, more time would be needed. To try and avoid staining

complications, a different antibody should be used. It would be worthwhile to look into other connexins located in the central nervous system to compare.

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