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Research Article

In-utero Exposure to Nicotine Alters the Response of the Cardiac Sodium Current to Isoproterenol; a Potential Mechanism for Sudden Infant Death Syndrome

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Abstract

Rationale: *In-utero* exposure to tobacco smoke is strongly associated to sudden infant death syndrome (SIDS) with cardiac arrhythmias considered the final cause of death. The mechanisms causing these arrhythmias remain largely unknown but seem to be linked to conduction anomalies and the inability of the newborn to accelerate cardiac rhythm at the onset of apnea.

Objectives: We previously reported that *in-utero* exposure to nicotine delays the development of the heart sinoatrial node and reduce its innervation for several weeks after birth in rabbits. Our goal with this study is to determine if exposing rabbit fetus to nicotine also altered the sodium current (INa) response to adrenergic stimulus in newborn kittens. Our general hypothesis is that a loss of adrenergic response prevents heart acceleration and awakening of newborn during sleep apnea.

Methods and results: Using electrophysiological techniques (patch clamp) on isolated cardiomyocytes we found that β-adrenoreceptor agonist isoproterenol increases the amplitude of INa by 50% in sham rabbits but had no effects in kittens exposed to nicotine *in-utero*. Analysis of the data also showed that nicotine abolished the increase in late sodium current by isoproterenol, an effect that will reduce the ability of newborn to increase their heart rate and may promote bradycardia. These results may in part explain the lack of arousal from sleep apnea in nicotine exposed infants and suggest that other arrhythmogenic mechanisms may also contribute to SIDS.

Conclusion: Our study provides the first evidence that nicotine may precipitate SIDS by altering the adrenergic response of the heart. This represents a new paradigm based on remodeling the cardiac electrical system as opposed to the current dogma of a centrally mediated respiratory deficit. The data also raise awareness on the use of nicotine replacement therapies for pregnant women.

ABBREVIATIONS

TGP: Tobacco Glycoprotein; ISO: Isoproterenol; SIDS: Sudden Infant Death Syndrome; HR: Heart Rate; HRV: Heart Rate Variability; APD: Action Potential Duratoin; NACh: Sodium Channel; IM: Intramuscular; SAN: Sinoatrial Node; GNa: Sodium Channel Conductance; INap: Peak Sodium Current; INaL: Late Sodium Current (sustained component)

INTRODUCTION

Sudden infant death syndrome (SIDS) is the leading cause of death in the first year of life. *In-utero* exposure to tobacco smoke is observed in 85% of SIDS cases and considered the highest risk factor [1,2]. Of the over 3000 known toxic and carcinogenic compounds found in tobacco smoke only tobacco glycoprotein (TGP) and nicotine were consistently linked to SIDS. While TGP triggers an anaphylactic response [3], only nicotine is associated to cardiac arrhythmias in newborns [4-10].

Evidence linked SIDS to a failed coordination of the cardiovascular and respiratory systems during the postnatal development of the heart thus causing cardiac arrhythmias and sudden death [11-13]. Among the hypothesis to explain SIDS is the failure of the newborn heart to accelerate at the onset of apnea. This cardiorespiratory reflex triggers awakening during sleep apnea in newborn babies and adults. As oppose to cardiac acceleration, the fetal heart responds to apnea by reducing its beating rate (bradycardia). This phenomenon, often referred as the diver reflex, is essential to minimize the consumption of oxygen in the womb [14]. However, during the postnatal period

when oxygen is freely available the bradycardic response to apnea is gradually replaced by the cardiorespiratory reflex and acceleration of heart rate (HR). This transition period in the early life of babies is characterized by large heart rate variability (HRV) and a higher risk of bradycardia and cardiac arrest in response to apnea.

HRV is maximum at 2-4 months of age and disappears in normal infants older than one year old. In resuscitated SIDS victims and pre-term babies HRV is more important [15-17], and this period of electrical instability is longer and often pushed toward the end of the first year [12,18]. In both cases bradycardia rather than HR acceleration develops in response to apnea [19,20], thus increasing the vulnerable period for developing arrhythmias or suffocating during sleep apnea. This inability to accelerate HR is a hallmark of the fetal heart diver reflex and suggests that postnatal development of the cardiac adrenergic response is delayed in pre-term newborns and SIDS babies exposed to nicotine *in-utero*. In agreement, our previous data indicate that nicotine delayed the development of the sinoatrial node in newborn heart [21].

SIDS is defined as sudden death without apparent anatomical defects of the heart. However, electrical instabilities within the conduction system are often detected and seem involved in SIDS fatal outcome [22]. Changes in cellular excitability, conduction and the cardiac action potential duration (APD) [23] are the most common cardiac occurrences in SIDS [24-27], and suggest the cardiac sodium current (INa) properties are altered. Indeed, INa is essential for the propagation of the electrical impulse from the sinoatrial node (SAN) to the ventricle and triggering the ventricular action potential. INa also modulates HR by contributing to diastolic depolarization in pacemaker cells of the SAN [28-32], and maintains the duration of the ventricular action potential [23,25-27]. Therefore, alterations in INa properties will modulate HRV and may slow heart rate. The correlation between electrophysiological functions of INa and the type of arrhythmias observed in SIDS strongly suggest a role for sodium channels (NaCh) and prompted us to examine how exposure to nicotine influence the response of INa to adrenergic stimulation in rabbit cardiomyocytes. We hypothesized that *in-utero* exposure to nicotine reduces the response of INa to β-adrenergic receptor agonist isoproterenol and, this loss of adrenergic response may prevent the cardiorespiratory reflex needed to awake from sleep apnea in SIDS victims.

Our data indicate that that i*n-utero* exposure to nicotine abolished the increase of INa amplitude by isoproterenol in cardiomyocytes of newborn kittens. This is likely to limit conduction and reduce the acceleration of sinus rhythm during adrenergic stimulation.

MATERIAL AND METHODS

All animal procedures, care, and maintenance were approved by the ethics review board of the Faculty of Medicine of the Université de Sherbrooke and follow the ARRIVE guidelines (Animal Research: Reporting of *In vivo* Experiments).

Animal model

New Zealand female rabbits (Charles River, QC, Canada) were mated and fertilization was confirmed by palpation of their abdomen during the first and second weeks of gestation. After 14 days of pregnancy, two osmotic pumps (2ML4, Alzet Osmotic Pumps, CA, USA) each containing 2 ml of nicotine solution (0.250 g/ml, Nicotine-Hydrogen Tartrate Salt, Sigma-Aldrich, ON, Canada) were inserted subcutaneously between the shoulder blades of the doe. The pumps diffused their content at a rate of 2.5 µ l/h for 28 days. Sham does were exposed to saline solution instead of nicotine. For pump implantation rabbits were anesthetized with a mix of 5% isoflurane (Abbott, QC, Canada) and pure oxygen. During surgery, isoflurane was reduced to 3% and the incision area was previously cleaned with 100% ethanol and a solution of iodine providione 10% (Rougier, QC, Canada). The incision was closed with stitches after implantation of the pumps.

Clinically, nicotine plasma concentration is calculated from titration of cotinine, its main metabolite. Serum concentration of cotinine in pregnant doe was monitored by a 0.5 ml draw from the ear marginal artery every 2 days. Blood samples were centrifuged and serum was tested using the ELISA-Cotinine quantification kit (Bio-Quant Inc., ON. Canada) according to the manufacturer's protocol. In kittens, cotinine was measured from a blood draw during heart excision.

Cardiomyocyte dissociation

Twenty six to 30 days after birth, rabbits were sedated (Atravet 0.25 mg kg IM) and heparinized (5000 U) for 30 minutes before being deeply anesthetized with sodium pentobarbital (25 mg / kg IV, Biomeda-MTC, ON, Canada) for surgery. The heart was quickly excised through an opening of the chest. Myocytes from the right atria were isolated by enzymatic dissociation and retrograde Langendorf perfusion as previously described [33,34]. Briefly, hearts were initially perfused with a calcium free Tyrode solution containing 2mmol/l EGTA and 0.1% of BSA. After 10 min, the solution was switched to a Tyrode solution containing 0.1 mmol/l of $CaCl_2$ and 230 U/ml of a type II collagenase (CLS 2, Worthington, Freehold, New Jersey) until isolated cells can be seen in small biopsies. Finally, the right atria was dissected out, minced and slowly stirred in a beaker containing the enzyme solution. The supernatant containing disassociated cells was stored at 4C in a cardioplegic (Krebs) solution containing (in mmol/l) : 100 potassium glutamate, 10 potassium aspartate, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 10 HEPES, 2% of BSA and 0.2 $CaCl_2$. Cardiomyocytes were tested within 8 hours of dissociation.

Electrophysiology

Myocytes were placed in a chamber mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) and were superfused with a solution containing (in mmol/l): 120 Choline-Cl, 10 NaCl, 5 NaOH, 2.8 Na Acetate, 4 KOH, 0.5 $CaCl_2$, 1.5 $MgCl_2$, 20 HEPES, 10 Glucose (pH 7.4 adjusted with NaOH). TetraEthylAmmonium chloride (TEA; 5 mmol/l) was added to this external solution to block TEA-sensitive K^+ currents and $CoCl_2$ (1 mmol/l), 4-AP (2 mmol/l) and $BaCl_2$ (5 mmol/l) were added to block ICaL, ITo and IK1 currents, respectively. INa was measured at room temperature (22°C) in the whole-cell configuration of the patch-clamp technique as previously described [35,36] using

an Axopatch 200B amplifier (Axon instruments, Union City, CA). Patch pipettes had resistance between 1 and 3 MΩ when filled with a solution containing (in mmol/l): 15 NaCl, 5 KCl, 120 CsF, 1.0 $MgCl_2$, 4 Na_2-ATP, 10 EGTA and 10 HEPES (pH 7.2 adjusted with CsOH). All solutions were adjusted to 300 mOsm with sucrose. Currents were filtered at 5 kHz and digitized at 10kHz. Whole cell capacitance and series resistance compensation (85%) were optimized to minimize the duration of the capacitive artifact and reduce voltage errors.

Data analysis

Data acquisition and analysis were performed using the pCLAMP program suite V9.2 (Axon instruments) and ORIGIN 8 (Microcal Software, Northampton, MA) software respectively. Activation and inactivation data were fitted to a standard Boltzmann distribution function:

$$Y = \frac{(A1 - A2)}{1 - \exp[(V_m - E)/V0.5] + A2}$$

where Y represents the fraction of activated (m) or available (h) obtained respectively from the ratio of the macroscopic conductance (GNa / GNa,Max) or the sodium current I / IMax. Vm: membrane test potential, E: sodium current reversal potential and V0.5 is the mid-potential for activation or inactivation. GNa was obtained from the current-voltage relationship as GNa= INa / (Vm - E) and GNa, Max represents the maximal Na^+ conductance (slope of the linear portion of the I/V relationship). Leak sodium current was calculated using the standard Hodgkin-Huxley formalism [37]: INa = m^3h^* GNa, Max * (Vm-E) where m and h are the fraction of activated and inactivated channels at voltage Vm.

Statistical analysis

Data are expressed as mean ± SEM. Significance was established by a Student T-test for paired or a T test for unpaired data and a Student F-test for differences between fit to data. Data were considered significantly different for p values < 0.05.

RESULTS

Light smokers maintain a plasma cotinine concentration between 100 and 150ng/ml [38]. Using the osmotic pump we were able to maintain a similar concentration during the last 21 days of gestation in rabbit doe (Figure 1).

Survival plots showed that 20% of newborn kittens exposed to nicotine died suddenly within the first 5 days of life (Figure 2A). Cotinine plasma level in kittens was 31 ± 7ng/ml at birth but rapidly decayed to 0 ng/ml at 30 days of age thus showing that fetuses were exposed to nicotine (Figure 2B). We therefore choose to make our electrophysiological measurements 30 days post-natal to test for long term alterations of INa.

The area surrounding the sinoatrial node is amongst the cardiac structures most extensively remodeled during postnatal development. This maturation process called resorptive degeneration [22,39,40], consists in gradual apoptosis of cells around the SAN and their replacement by less excitable atrial cardiomyocytes. Since cellular excitability is highly dependent of the activity of INa, we tested if nicotine altered its response to adrenergic stimulus in cardiomyocytes from the right atrium close to the SAN. In sham myocytes superfusion with 10µmol/l isoproterenol increased INa maximum amplitude by 91 ± 9% (Figure 3A). Analysis of the current-voltage relationship (I/V) showed that isoproterenol shifted the activation threshold of INa from -60mV in control to -70mV (Figure 3B). Maximum INa amplitude voltage was similarly shifted from -30mV in control to -45 mV following superfusion with isoproterenol. Analysis of the maximum conductance (Figure 3E) obtained as the slope of the linear portion of the I/V curve (Figure 3B) revealed that isoproterenol did not change GMax density with values of 2.9 ± 0.2 pS/pF and 3.1 ± 0.2 pS/pF in control myocytes and following exposure to isoproterenol. Since GMax represents the maximum number of channels available this result suggests that the increase in INa amplitude in sham myocytes is due to alterations in the gating of the NaVs rather than recruitment of new channels. This was confirmed by analysis of the activation curve (Figure 3F)

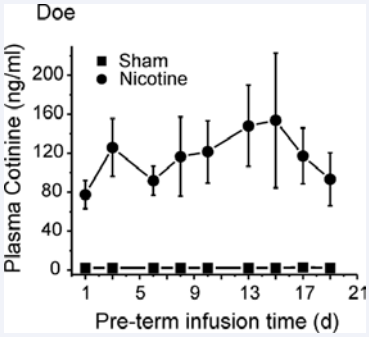

Figure 1 Blood cotinine concentration in gestating doe. Cotinine concentration was measured by ELISA (see methods) on blood samples taken at indicated ages (post-partum days).

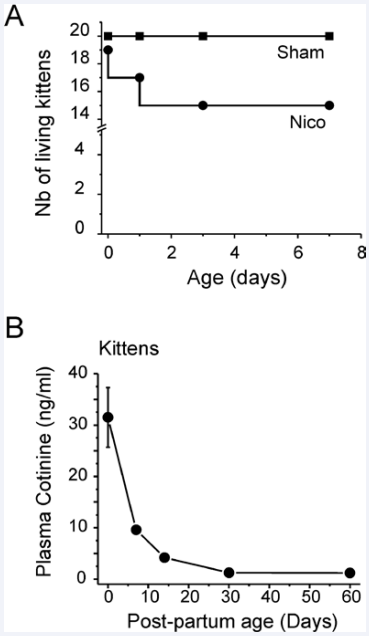

Figure 2 Kaplan Meier survival plot for kittens exposed to nicotine in-utero (A) and plasma concentration of cotinine in kitten after birth (B).

which showed that isoproterenol shifted the voltage at which INa reached 50% of its maximum value (V0.5) from -37.5 ± 0.2 mV in control to -51.7 ± 0.5 mV.

INa maximum amplitude in cardiomyocytes from nicotine exposed kittens was 48 ± 9% higher vs sham cells (Figure 3A,D). INa threshold for activation was -70mV in nicotine exposed myocytes and reached maximum amplitude at -40 mV (Figure 3C). These voltages are similar to those found in sham myocytes under isoproterenol. Maximal conductance was similar between sham and nicotine exposed cells and was not altered by isoproterenol (Figure 3E). In nicotine exposed cardiomyocytes, adrenergic stimulation did not increase INa (Figure 3D) nor did it change its activation threshold or maximum amplitude voltage (Figure 3C,D). As a result, INa remained 24% smaller compared to values in sham myocytes under the same conditions (Figure 3D). This loss of sensitivity to isoproterenol was confirmed by the absence of significant variation in the mid-activation potential of INa under isoproterenol (Figure 3G).

INa maximum amplitude is determined by the total number of Na channels available for opening (steady-state inactivation). Availability of the channels was measured using a standard inactivation protocol (Figure 4A). Nicotine negatively shifted steady-state inactivation of INa with 50% of the channels available at voltages of -81.3 ± 0.2 mV and -85.3 ± 0.1 mV in sham and exposed myocytes respectively ($p<0.05$, *F*-test). Isoproterenol shifted mid-voltage of inactivation to -85.3 ± 0.2 mV in sham ($p<0.05$) but had no effect on nicotine exposed myocytes (V1/2: -84.1 ± 0.1 mV). There was no difference between the sham and nicotine exposed myocytes following application of isoproterenol.

Steady-state activation curves determine the probability of opening of the channels upon stimulation while steady-state inactivation represents the fraction of the population available at a given resting voltage. The overlap between the steady-state activation and inactivation curves (Figure 5), thus creates a voltage range where Na^+ channels transit spontaneously between the open and inactivated states and generate the so-called window current (IW). In sham animals, isoproterenol shifted this overlap towards more negative potentials resulting in a higher probability for the channel to open spontaneously at negative potentials. Surprisingly, nicotine exposure shifted this overlap to a larger extent than isoproterenol did in sham animals. Moreover, isoproterenol had depolarizing effect in nicotine exposed cardiomyocytes by shifting the overlap towards more positive potentials.

IW can be calculated from our measurements as the product of the fraction of channels available, their opening probability and the maximum conductance using the standard Hodgkins-Huxley model [37]. The model predicted that in sham myocytes, isoproterenol will increase the amplitude of IW by 332% (4.6 folds) and shift its peak value from -35 mV to -47 mV (Figure 6A). Nicotine exposure alone increased the amplitude of IW by 325 % (4.5 folds). As opposed to its effect in sham myocytes, isoproterenol reduced the maximum amplitude of IW by 54 % and shifted its voltage towards more positive potentials following exposure to nicotine (Figure 6B).

To test the predictions of the model we measured the amplitude of the late sodium current (INaL) once fast inactivation of the current is completed and the residual Na^+ current is dominated by IW (Figure 7A). In agreement with the model we found that isoproterenol shifted the I/V relationship towards more negative potentials and increased the maximum amplitude

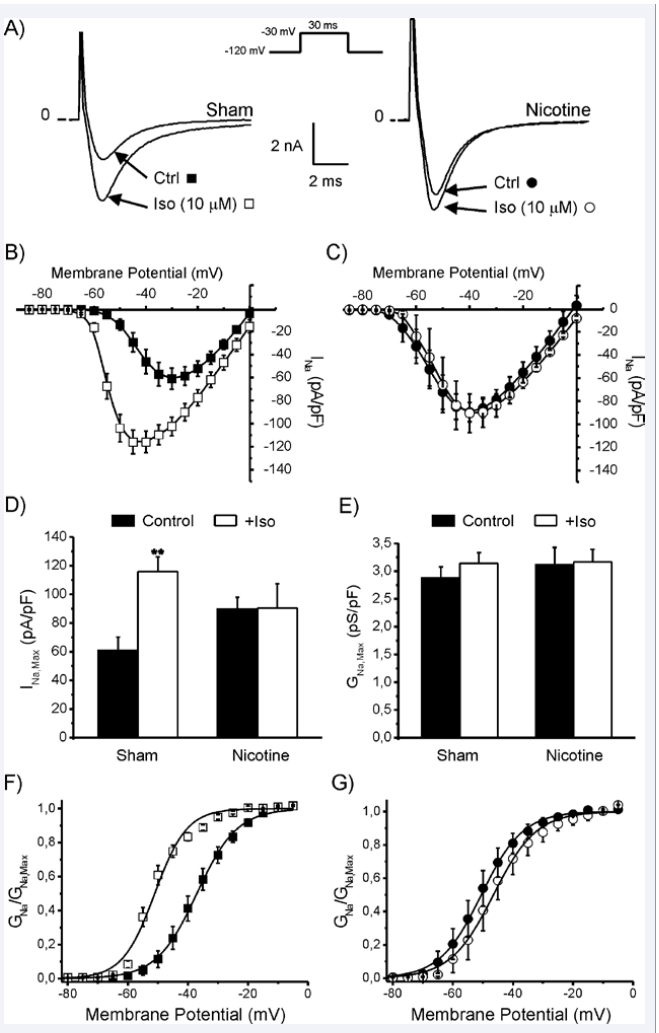

Figure 3 *In-utero* exposure to nicotine abolished the effect of ß–adrenergic stimulation on the cardiac sodium current (INa). A. Representative sodium current recordings (INa) in cardiomyocytes isolated from the right atrium of sham and kitten exposed to nicotine *in-utero* in control (Ctrl) and after perfusion with isoproterenol (+Iso), INa was elicited by 30-ms test pulses from a holding potential of -120 mV (insert), B, C. Current -voltage relationship (I/V). Peak INa was normalized to the capacitance of their respective cells and plotted as current density (pA/pF) for each test potential. Number of cells sham (n=8), +Iso (n=9) and nicotine (n=8), +Iso (n=6) conditions. D. Maximum current density (from panels B and C). E. Isoproterenol did not increase the maximum conductance (GNa,Max) of INa. Conductance (GNa) was calculated as the ratio INa/(Vm-ENa) where the denominator represents the driving force for the current and ENa and Vm are the reversal potential for the current (from panels B and C) and the test voltage respectively. GNa therefore represents the fraction of channels activated at each voltage. F. Isoproterenol (open symbols) shifted the mid activation potential (V0.5) of sham myocytes from -37.5 ± 0.2 mV to - 49.5 ± 0.9 mV ($P<0.05$, F-test) but did not in nicotine-exposed (filled symbols) animals (-53.4 ± 0.3 and -46.3 ± 0.4 mV). Data are presented as means ± SEM. **$P<0.01$, T-test.

of INaL by 260 ± 15 % (3.6 fold) in sham myocytes (Figure7B, D). Nicotine by itself increased INaL maximum amplitude by 175 ± 15 % (2.5 folds) and hyperpolarized the I/V relationship by 15 mV compared to sham myocytes (Figure 7C, D). Isoproterenol had no significant effect on INaL in cardiomyocytes exposed to nicotine (Figure 7C). However the overall response to isoproterenol was 41 ± 5 % smaller in nicotine exposed cardiomyocytes vs sham.

We next sought to determine if nicotine altered the fraction of channels contributing to INaL. Figure 7 shows that isoproterenol increased the fraction of peak current (INap) contributing to INaL in sham myocytes. In contrast, the fraction of channels contributing to INaL in nicotine exposed cardiomyocytes remained constant.

CONCLUSIONS

Our model reproduced the conditions associated to nicotine and SIDS. We found that plasma concentration of cotinine in our model was similar to the one observed in human smokers and sudden death in newborns occurred more frequently in nicotine exposed kittens as expected in SIDS [1,2]. One of the main findings from this study is that *in-utero* exposure to nicotine completely

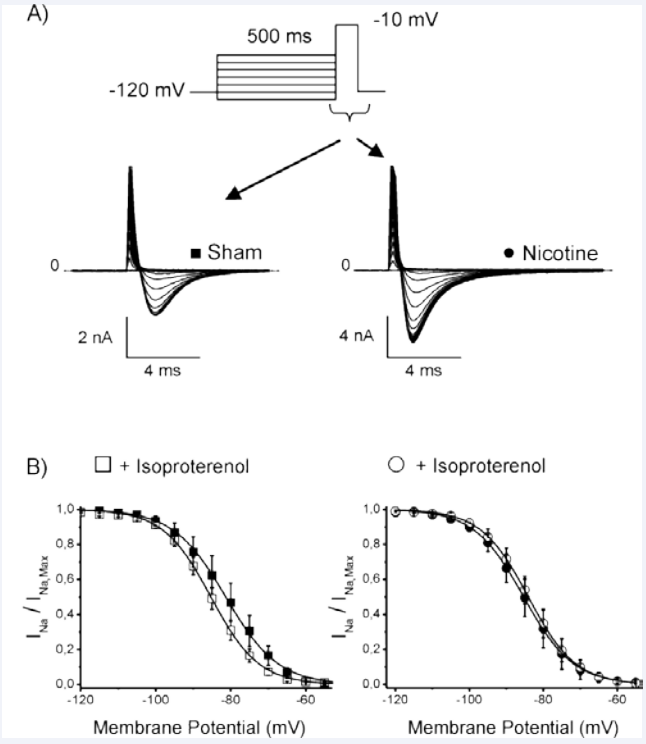

Figure 4 Nicotine blunted the effect of isoproterenol on sodium channels availability. A. Representative current recordings during a test pulse to -10 mV preceded by conditioning pulses from- 140 mV to +40 mV in increments of 5 mV from a holding potential of -120 mV (inset). B. Inactivation curves (availability) in sham (n=7), +Iso (n=8) and nicotine (n=8), +Iso (n=7) conditions were obtained by plotting the ratio of INa to its maximum value against the conditioning pulse voltage. Data were fitted to a Boltzmann distribution function. Isoproterenol significantly shifted mid-inactivation potential (V0.5) from -81.3 ± 0.2 to -85.3 ± 0.2 mV (P<0.05, F-test) in sham cardiomyocytes but had no effect in nicotine-exposed animals (-85.3 ± 0.1 and -84.1 ± 0.1 mV). Data are expressed as mean ± SEM.

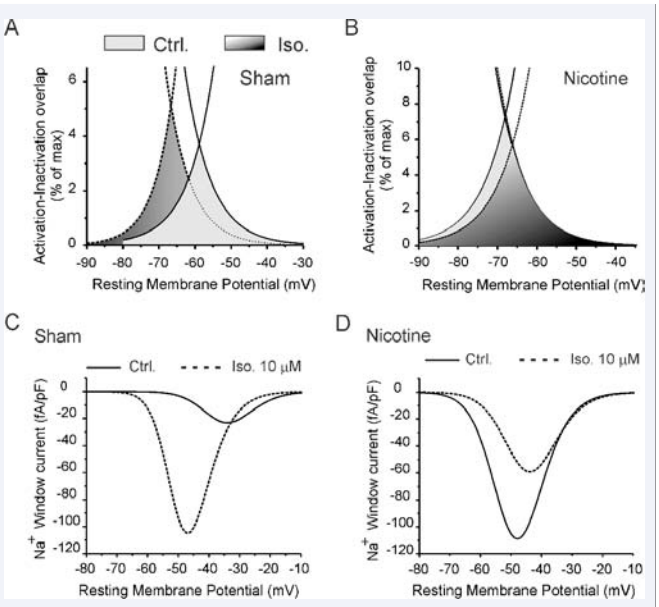

Figure 5 Exposure to nicotine blunted the effect of isoproterenol (Iso) on the voltage range where the activation and availability of the channels overlap (A, B) and reversed the response of the sodium leak (window) current (C-D), Activation (Figure 3E, F) and inactivation (Fig 4B) curves obtained from the Boltzmann distribution fit to data are enlarged to display the voltage range where each curve overlap in sham and nicotine exposed cardiomyocytes respectively. Window currents were calculated from the overlap of the activation and inactivation curves using the standard Hodgkins and Huxley formalism (see methods).

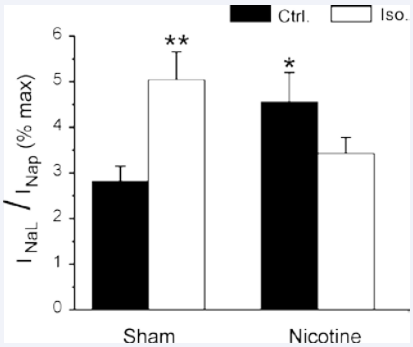

Figure 6 Modulation of the late sodium current (INaL) by isoproterenol reflects the dependence on voltage predicted by the window current (Figure 5). A. Representative INa recordings from a cardiomyocyte exposed to nicotine in-utero following a series of voltage pulses in 5 mV increments from a holding potential of -120 mV (protocol in inset). The arrow indicates measurement of the late current (INaL) once fast inactivation of the channels is completed. B, C. Current-voltage relationship of INaL in cardiomyocytes isolated from sham (n=9) and nicotine exposed kittens respectively (n=6) in control and following perfusion with isoproterenol (10 µmol/l). Nicotine abolished the response to isoproterenol. D. Maximum late Na+ current density INaL, max. Statistical significance: *: p < 0.05, **: p < 0.01, ***: p < 0.001 (*: vs sham control), †: p < 0.05 (vs sham Iso.). ANOVA.

abolished the increase of INa by isoproterenol. This loss of adrenergic response is not without significant consequences. Indeed the ability to adjust INa amplitude and activation

threshold to the adrenergic tone is essential to meet requirements of the heart. In newborn, this adaptation is responsible for the sympathetic discharge triggering the cardio-respiratory reflex at the onset of apnea. Without a sympathetic response of INa opposing the vagal discharge causing the ''diver reflex'' excitability will not increase and heart rate will gradually slow down. This bradycardic response is often observed in resuscitated SIDS infants and premature babies. While multiple causes for the initial sleep apnea vary from upper airway and laryngeal reflexes [41], to immune response to viral infections [42,43], the final step in SIDS is invariably bradycardia and cardiac arrest. Nicotine may interfere with various centrally mediated processes controlling breathing patterns or interfere with ventilator chemo- or mechanical reflexes preventing death during apnea. However, in this study we show that nicotine inhibits the cardiac adrenergic response needed for the cardiorespiratory reflex. Moreover, we found that prenatal exposure to nicotine induced a long lasting increase of peak INa amplitude at baseline. This remodeling of the cardiac electrical system represents a significant departure from the general view of respiratory dysfunction as the primary cause of SIDS [44]. In this new paradigm, the loss of adrenergic sensitivity may destabilize the cardiac response to apnea. As a consequence benign apnea that would normally trigger a sympathetic response leading to arousal may become life threatening during sleep. This last effect may be exacerbated by precipitating factors such as airway infections or chemicals interfering with respiration.

Our data showing that nicotine increases IW suggest other arrhythmogenic mechanisms may also contribute to SIDS. IW and the Na/Ca exchanger (NCX) act in concert to maintain intracellular Na^{+} and Ca^{++} homeostasis. IW is acting as a leak current to modulate the sodium gradient between the intra- and extracellular milieu which, in turn, regulates the rate at which NCX extrudes calcium entering the cell during each action potential. During a transient adrenergic stimulation an increase in IW like the one we observed in sham myocytes is beneficial as it will promote contraction of the ventricle and increase excitability by depolarizing the resting membrane potential for a short period of time. However, nicotine has quintupled the amplitude of IW a result confirmed by a 4 fold increase in INaL. This larger IW will chronically increase the concentration of intracellular calcium, strongly depolarize the resting membrane potential of cardiac cells and potentially prolong the duration of the action potentials. One can therefore expect significant changes in cardiac excitability and repolarization.

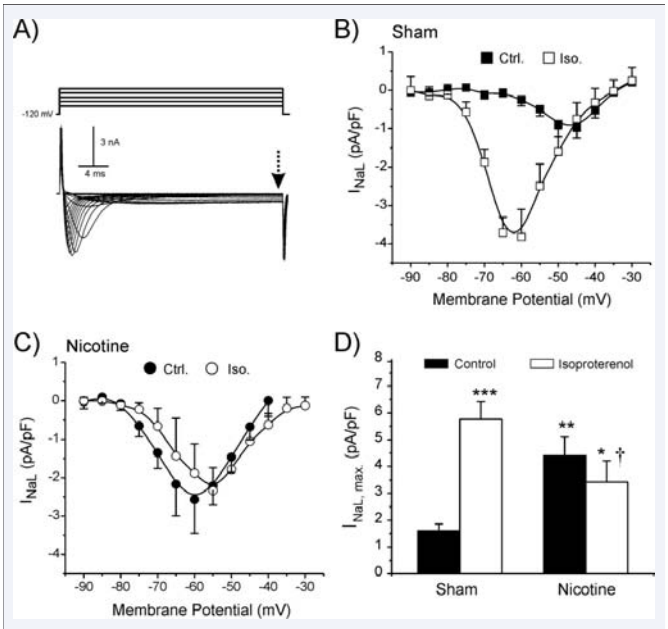

Figure 7 Fraction of channels contributing to INaL expressed as % of the maximum peak current (INap) shown in Fig. 3 (B, C). Statistical significance: *: $p < 0.05$, **: $p < 0.01$ (vs sham Ctrl). ANOVA.

The contribution of the late sodium current to repolarization and QT prolongation is well established [24,26, 27,45,46]. A first link between INaL and SIDS was provided by association of a mutation in the cardiac sodium channel gene SCN5A to a near-miss case in 2000 [23]. Since then, most efforts to associate SIDS with QT prolongation have focused on identification of mutations in channels involved in cardiac repolarization. However, our results on INaL indicate that QT prolongation after *in-utero* exposure to nicotine may also occur in absence of ion channel mutations. Moreover, our data indicate that the effects of adrenergic stimulation on INaL are reduced following exposure to nicotine. This last result confirms in part the initial mechanism proposed independently by Schwartz [47] and Maron et al. [48], as early as 1976 whereby changes in the adrenergic response or catecholaminergic innervation of the heart were responsible for the QT prolongation observed in SIDS.

Our results may also help explain the particular period when infants are most vulnerable to SIDS. In human the QT interval gradually prolongs and peaks at 6 to 11 weeks in infancy [49], before shortening to childhood level. This maturation period overlaps with the most vulnerable period for SIDS (2 months) thus suggesting that both events are related. Considering that exposure to nicotine quadrupled the amplitude of INaL, we speculate that a similar augmentation in human ventricle would add to the natural prolongation of the action potential and could bring the QT interval to arrhythmogenic levels during the peak of the electrophysiological maturation process.

Excessive prolongation of the duration of the action potential in atrium or in the ventricle is linked to calcium overload and triggered activity. During calcium overload, spontaneous electrical currents start to appear during repolarization of the cardiac cells and may trigger spurious action potentials. Our data thus raise the possibility that nicotine, by increasing INaL, may promote triggered activity and fibrillation in the atrium. However, isoproterenol may have protective effects in this condition. Indeed, we found that isoproterenol reduced IW amplitude in nicotine exposed kittens. In agreement, we found that isoproterenol had a tendency to reduce INaL amplitude and shifted its peak amplitude towards more depolarized potentials, the latter promoting a further reduction in INaL at resting membrane potentials ~ -70 mV – -60 mV. By reducing the contribution of INaL, sympathetic stimulation may lower intracellular calcium and have protective effects against triggered activity in nicotine exposed infants. We speculate this mechanism may promote sudden infant death during sleep, at a time when

the protective effect of the sympathetic activity is reduced, rather than during awake stifling. In the ventricle, triggered activity caused by an increase in INaL could generate Torsade de Pointes (TdP) arrhythmias [50]. TDP onset is rapid and occurs with little or no warning making it the most unpredictable and lethal arrhythmia.

Our data also show that nicotine hyperpolarized the threshold for activation of INa close to the normal resting membrane potential of cardiomyocytes. This effect combined with the depolarization of the resting membrane potential brought by a larger IW will further reduce the voltage gap between the resting voltage of the cell and INa threshold, thus making cardiomyocytes hyperexcitable and the heart prone to tachyarrythmias or atrial fibrillation. This hypothesis is supported by observations of large heart rate variability in resuscitated SIDS infants [51,52], indicative of alterations in the adrenergic regulation and hyperexcitability. However the effects of the larger INaL and its displacement toward more negative potential by nicotine are likely to have more impact on the more depolarized resting membrane potential of cells within the SAN.

Our results on INaL are consistent with the observation of a larger Na^+ window current modulating the firing rate of the SAN in newborn kittens but not in adult rabbit [31]. They also provide a potential mechanism to explain why SIDS infants do not wake-up at the onset of apnea. In sham myocytes, isoproterenol increases the amplitude of INaL thus accelerating the diastolic depolarization of pacemaker cells and sinus rhythm. This may contribute to the cardiorespiratory reflex. However, in nicotine exposed SAN myocytes, a chronic increase of INaL is more likely to depolarize the maximum diastolic potential thereby reducing the amplitude of the pacemaker current If and inactivating the calcium current responsible for the onset the action potential. In this case, diastolic depolarization will be slower thus lengthening the time between action potentials and promoting bradycardia. Moreover, this bradycardic effect cannot be compensated by an increase in adrenergic tone since INaL is insensitive to isoproterenol in nicotine exposed myocytes. Therefore centrally mediated adrenergic bursts to accelerate heart rate at the onset of apnea are likely to be less efficient and the intensity of the cardiorespiratory reflex will be attenuated. The loss of cardiac response is likely to be amplified in the settings of enhanced vagal tone such as during sleep or during respiratory infections. This may in part explain some of the conduction anomalies and the bradycardia observed in SIDS infants exposed to nicotine *in-utero* [22].

Our results (Figure 7) show that INaL amplitude does not increase proportionally to INap when sham cardiomyocytes are stimulated by isoproterenol. This result indicates that activation of the G protein-coupled β-adrenergic receptors triggered the activation of intracellular pathways that act separately on the peak and late Na^+ current. This effect was not observed in nicotine exposed myocytes where the fraction of late/peak current remained constant. This suggests that nicotine blunts the overall activity of the β-receptors rather than targeting a specific intracellular pathway in the cardiomyocytes. One potential explanation for the phenomenon is that chronic stimulation of adrenergic neurons by nicotine created conditions of sympathetic dominance resulting in gradual desensitization of β-adrenergic receptors [53]. Alternatively, nicotine may also directly modulate sodium channel activity. At this point we cannot distinguish between the two mechanisms. Nonetheless, the augmented INa and INaL amplitude in nicotine exposed cardiomyocytes and their lack of response to adrenergic stimulus strongly suggest that arrhythmogenic mechanisms traditionally linked to β-adrenergic overstimulation participate to SIDS.

Finally, our results clearly demonstrate that *in-utero* exposure to nicotine interfere with cardiac excitability and that the ensuing remodeling of the heart electrical system induce several potentially arrhythmogenic changes that could be linked to SIDS. Our data thus raise awareness on the use of nicotine replacement therapies in pregnant women.

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