Alterations in cholinergic sensitivity of respiratory neurons induced by pre-natal nicotine: a mechanism for respiratory dysfunction in neonatal mice

Claudio Coddou†, Eduardo Bravo‡ and Jaime Eugenín*

Faculty of Chemistry and Biology, Department of Biology, Universidad de Santiago, USACH, Chile, Casilla 40, Correo 33, Santiago, Chile

Nicotine may link cigarette smoking during pregnancy with sudden infant death syndrome (SIDS). Pre-natal nicotine leads to diminished ventilatory responses to hypercarbia and reduced central chemoreception in mice at post-natal days 0–3. We studied how pre-natal nicotine exposure changes the cholinergic contribution to central respiratory chemoreception in neonatal isolated brainstem–spinal cord and slice preparations.

Osmotic minipumps, implanted subcutaneously into 5–7 days pregnant mice, delivered saline or nicotine ditartrate 60 mg kg⁻¹ d⁻¹ for up to 28 days. In control preparations, acidification of the superfusion medium from pH 7.4 to 7.3 increased the frequency and reduced the amplitude of fictive respiration. In nicotine-exposed neonatal mice, the reduction in amplitude induced by acidification was reduced. In control preparations, atropine suppressed respiratory responses to acidification, while hexamethonium did not. By contrast, in nicotine-exposed preparations, hexamethonium blocked chemosensory responses but atropine did not.

Our results indicate that pre-natal nicotine exposure switches cholinergic mechanisms of central chemosensory responses from muscarinic receptors to nicotinic receptors. Modification of the cholinergic contribution to central chemoreception may produce respiratory dysfunctions, as suggested by receptor-binding studies in victims of SIDS.

Keywords: central chemoreception; respiratory rhythm generator; sudden infant death syndrome; hypercarbia; muscarinic receptors; nicotinic receptors

1. INTRODUCTION

Cholinergic inputs provide excitatory drive to neurons of the respiratory pattern generator (Weinstock 1981; Murakoshi et al. 1985; Gillis et al. 1988; Nattie & Li 1990; Burton et al. 1994, 1995; Shao & Feldman 2000, 2001, 2005; Hatori et al. 2006) and play a part in central chemosensitivity to H⁺ and PCO₂ (Dev & Loeschcke 1979a,b; Fukuda & Loeschcke 1979; Nattie et al. 1989; Monteal et al. 1990; Burton et al. 1997; Eugenin & Nicholls 1997). The sites of acetylcholine action overlap with CO₂-chemosensitive regions, and the responses elicited by acetylcholine are similar to those elicited by low pH stimulation (Issa & Remmers 1992; Eugenin & Nicholls 1997). In addition, blockade of muscarinic receptors, which are expressed in various brainstem respiratory regions (Kinney et al. 1995a,b; Mallios et al. 1995), reduces respiratory responses to acidosis (Dev & Loeschcke 1979a,b; Monteal et al. 1990; Eugenin & Nicholls 1997).

If acetylcholine plays a neurotrophic role during brain development (Lauder & Schambra 1999; Gu 2002), treatment of a foetus with nicotine (a teratogen as well as an acetylcholine agonist) might give rise to changes in the properties of brainstem chemoreceptors in the neonatal animal (Mitchell et al. 1993; Kohlendorfer et al. 1998; Chong et al. 2004; Slotkin 2004). Indeed, there is evidence that links sudden infant death syndrome (SIDS), a cause of death in infants under 1 year old in developed countries, with smoking during pregnancy (Dwyer & Ponsonby 1995; Slotkin 1998). SIDS in turn could be due to abnormalities in the generation of the respiratory rhythm or its modulation by chemosensory input (Nattie & Kinney 2002; Eugenin et al. 2008). In fact, infants who died from SIDS had previously shown alterations of their breathing patterns during sleep (Schechtman et al. 1991; Kahn et al. 1992). Moreover, infant victims of SIDS or infants born of mothers who smoked showed a high incidence of central respiratory dysfunctions (Brady & McCann 1985), a major number of central apnoeas (Gennser et al. 1975; Kahn et al. 1988; Schechtman et al. 1991), diminished chemoreflexes (Shannon et al. 1977; Ueda et al. 1999) and decreased spontaneous and evoked arousability (Newman et al. 1997a,b; Monteal et al. 1990; Eugenin & Nicholls 1997).

*Author and address for correspondence: Laboratory of Neural Systems, Department of Biology, Universidad de Santiago de Chile, USACH, Casilla 40, Correo 33, Santiago 1, Santiago, Chile (jaime.eugenin@usach.cl or jeugenin@gmail.com).

†These authors contributed equally to the study.

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Neonatal rats and mice that were exposed to nicotine during pregnancy showed hypoventilation and increased frequency of apnoea (St John & Leiter 1999; Robinson et al. 2002; Huang et al. 2004; Eugenin et al. 2008). Pre-natal nicotine also impairs hypoxia-induced autoregulation from primary apnoea in neonatal rats (Fewell et al. 2001) and hypoxia- or hypercarbia-induced ventilatory reflexes in neonatal mice, awakening rats and sleeping lambs (St John & Leiter 1999; Hafstrom et al. 2002; Huang et al. 2004; Simakajornboon et al. 2004; Eugenin et al. 2008).

Recently, using brainstem–spinal cord preparations, we have shown that pre-natal nicotine reduces central respiratory chemosensitivity in neonatal mice (Eugenin et al. 2008). Since pre-natal nicotine can affect the expression of acetylcholine receptors (Slotkin 1998), a reduction in central chemoreception might be due to changes in cholinergic chemosensory drive. Previous results using the ‘en bloc’ brainstem–spinal cord preparation indicate a change in cholinergic contribution to central chemoreception (Eugenin et al. 2008). To explore this possibility in more detail, we studied the effects of pre-natal nicotine exposure upon the cholinergic tonic drive of fictive respiration and upon the muscarinic and nicotinic receptor contributions to the respiratory central chemoreception. We further compared the effects of nicotine on en bloc brainstem–spinal cord and slice preparations.

2. METHODS

(a) Preparations

Fifteen adult CF1 mice, 5–7 days pregnant, were anaesthetized with ketamine/xylazine (80/20 mg kg\(^{-1}\) i.p.; Troy Laboratories, Smithfield, Australia and Alfasan International, Woerden, The Netherlands). Under strict aseptic conditions, subcutaneous implanting of 28-day osmotic minipumps (2004, Alzet, Cupertino, CA, USA) delivering saline (controls, \(n = 5\)) or nicotine bitartrate (60 mg kg\(^{-1}\) d\(^{-1}\), \(n = 10\)) at a rate of 0.25 \(\mu\)l h\(^{-1}\) was performed through an incision made between the scapulae. As described previously (Eugenin et al. 2008), osmotic minipumps allow one to distinguish between the effects of nicotine itself in the foetus from those caused either by the stress of daily injections (Suemaru et al. 1992; Houdi et al. 1995) or by possible foetal hypoxia–ischaemia owing to uterine vessel vasoconstriction caused by the peak of plasmatic nicotine achieved during injections. Mice were maintained in separate cages with water and food ad libitum at 22 °C under a 12 L : 12 D cycle. At the end of the experiments, animals were sacrificed with an anaesthetic overdose.

(b) Recording fictive respiration in en bloc brainstem–spinal cord preparations and brainstem slices

In order to evaluate whether pre-natal nicotine exposure affects chemosensory responses in a reduced preparation containing the preBötzinger complex (preBötC) (Smith et al. 1991), experiments were performed in slices and compared with en bloc brainstem–spinal cord preparations that also contained the pre-inspiratory parafacial respiratory group (Onimaru et al. 2006).

Experiments were carried out in 96 newborn animals (P0–P6), anaesthetized with methohexital and cooled on ice. The central nervous system was removed, decerebrated through a ponto-bulbar transection and immersed in artificial cerebrospinal fluid (aCSF) containing (in mM): 125.0 NaCl, 5.0 KCl, 24.0 NaHCO\(_3\), 1.25 KH\(_2\)PO\(_4\)•H\(_2\)O, 0.8 CaCl\(_2\), 1.25 MgSO\(_4\)•7H\(_2\)O (Sigma, St Louis, MO, USA), 30.0 D-glucose (Merck, Darmstadt, Germany) and equilibrated with \(O_2:\ CO_2 = 95\% : 5\%\) per cent : 5 per cent (pH 7.40) at 4 °C. For en bloc preparations, the isolated tissue constituted by the brainstem and the spinal cord was transferred to a recording chamber 2 ml in volume and superfused with aCSF at 25 °C. A thin film partition, sealed with Vaseline at C1–C2, allowed us to superfuse the brainstem separately from the spinal cord with a continuous flow of aCSF (0.8–2.0 ml min\(^{-1}\)). For the slice preparation, the brainstem was mounted on agar, and a 700 \(\mu\)m slice containing the preBötC was obtained using a vibratome as described previously (Pena & Ramirez 2004). Slices were transferred to a 0.5 ml recording chamber with a continuous flow of 1.0–2.0 ml min\(^{-1}\).

(c) Electrical recording

Spontaneous activities from C3–C5 ventral roots in en bloc preparations or from the ventral respiratory group (VRG) in slices were recorded using glass suction electrodes at 24–25 °C. Under stereomicroscopic vision, the tip of the glass electrode driven by a three-axis micro-manipulator was placed in close contact with the caudal surface of the slice in the region of the VRG. Electrical signals were amplified by a low-noise differential amplifier (Grass, Model P55), integrated with a full-wave rectifier (time constant = 100 ms), displayed on an oscilloscope (VC 6041i, Hitachi, Japan) and recorded and analysed with an Axoscope-Digipack 1320A AD acquisition system (Axon Instruments, Union City, CA, USA).

(d) Acidic stimulation

The pH of the brainstem superfusion medium (7.3 and 7.4) was obtained by equilibrating aCSF prepared with different final concentrations of sodium bicarbonate (19 and 24 mM, respectively) with 95 per cent \(O_2\) 5 per cent \(CO_2\). Reduction in sodium bicarbonate from 24 to 19 mM was compensated by increasing the final concentration of NaCl to 130 mM to eliminate any osmotic effect. In previous work, we showed that, in the range of concentration used here, changes in [NaCl]\(_0\) did not alter fictive respiration (Eugenin et al. 2006). In addition, changes in pH were obtained by switching the equilibrating gas mixture from 95 per cent \(O_2\) and 5 per cent \(CO_2\) (pH 7.4) to 90 per cent \(O_2\) and 10 per cent \(CO_2\) (pH 7.2). The tip of a micro-combination pH electrode (Model 9811, Orion, Beverly, MA, USA) was placed in the recording
chamber and connected to a pH/ion amplifier (Model 2000, A-M Systems, Everett, WA, USA) to record the pH of the superfusion medium. Before the evaluation of the effects of acidification, ventral root activity had to be regular and stable for at least 3 min.

(e) Evaluation of cholinergic contribution to chemosensitivity and of cholinergic drive upon fictive respiration
Cholinergic contribution to central chemosensitivity was evaluated in en bloc and slice preparations. Fictive respiration was recorded while superfusion medium was switched from pH 7.4 to 7.3 in the presence or absence of aCSF containing muscarinic acetylcholine receptor blocker, atropine 100 µM (Sigma) or nicotinic acetylcholine receptor (nAChR) blocker, hexamethonium chloride 100 µM (Sigma). After pH 7.3 stimulation, the superfusion was returned to pH 7.4 and a recovery recording was performed.

Cholinergic drive of fictive respiration was evaluated in en bloc and slice preparations, by measuring the effects of muscarinic and nAChR blockers on the basal fictive respiration. Tonic actions of endogenous acetylcholine release were evaluated through the effects of an acetylcholinesterase blocker, neostigmine 100 µM, upon fictive respiration.

(f) Data analysis
Neonates in each experimental group were obtained from at least three to four litters. The amplitude of a burst of action potentials was estimated in vitro from the difference between the peak value of the integrated signal and the value of the integrated activity immediately before the onset of the burst, expressed in arbitrary units. Cycle duration was measured from the onset of one burst of action potentials to the onset of the next. Duration of the burst, which corresponded to the inspiratory duration for the respiratory-like rhythm, was measured from the onset to the offset of the burst. Instantaneous rhythm frequency was calculated from the reciprocal value of the cycle duration and expressed as bursts per minute. Values were expressed as mean ± s.e.m.

The statistical significance of differences induced by treatments (acidification and cholinergic drugs) was ascertained using a two-tailed p-level estimated through a Wilcoxon signed-rank test. Differences in the magnitude of responses between control and nicotine-exposed preparations were assessed with Student’s t-test for independent samples. Comparison of multiple independent groups was performed using a two-tailed p-level estimated through ANOVA followed by Bonferroni post hoc test. Rejection of the null hypothesis was done if p < 0.05.

3. RESULTS
(a) Basal activity and responses to acidification
In control neonatal mice, spontaneous rhythmic activity recorded from en bloc preparations consisted of bursts of action potentials appearing rhythmically at a frequency of 9.7 ± 0.7 bursts min⁻¹ (ranging from 3 to 18 bursts min⁻¹), with a duration of 0.91 ± 0.03 s (n = 15, figure 1a). In control slices, bursts of action potentials recorded from the surface of the VRG appeared rhythmically at a frequency of 11.3 ± 1.4 bursts min⁻¹ and lasting 0.87 ± 0.03 s (n = 8, figure 1b). No significant differences in these parameters were found between en bloc and slice preparations.

Acidification of the superfusion medium induced changes in fictive respiration in control en bloc (P0–P3) and slice (P1–P6) preparations. As previously described (Infante et al. 2003; Eugenin et al. 2006), acidification of the brainstem superfusion medium from 7.4 to 7.3 reduced the amplitude of the integrated inspiratory burst and increased the frequency of the fictive respiration in en bloc preparations (figure 1a,c,d). In slices, a similar pattern of response was observed: an increase in respiratory frequency (figure 1b,d) and a decrease in the amplitude of the integrated burst (p < 0.01, Wilcoxon test). However, the reduction in amplitude was significantly lower than that observed in en bloc preparations (figure 1c, p < 0.01, unpaired t-test).

(b) Effects of pre-natal nicotine upon basal activity and acidification responses
Basal frequency of fictive respiration in en bloc preparations from nicotine-exposed mice was 7.0 ± 1.1 bursts min⁻¹ (n = 10), which was lower than that found in controls (p < 0.01, unpaired t-test). The duration of the inspiratory burst was 0.97 ± 0.09 s (figure 2a) and not different from control. Contrary to en bloc preparations, slices from nicotine-exposed mice showed a higher basal frequency than that observed in control slices (14.6 ± 1.5 bursts min⁻¹, n = 9, p < 0.01, unpaired t-test, figure 2b). The cycle duration in slices from nicotine-exposed mice was 0.85 ± 0.04 s.

As illustrated in figure 2c,d, in en bloc preparations from nicotine-exposed mice, acidification induced a decrease in the amplitude and an increase in the frequency (p < 0.01, Wilcoxon test). In slice preparations, acidification induced only a frequency increase but not an amplitude decrease (figure 2c,d). The increases in the basal frequency of fictive respiration induced by acidosis (figure 2a,b,d) in en bloc and slice preparations from nicotine-exposed mice were similar to those observed in controls. However, the reductions in amplitude were smaller in en bloc preparations obtained from nicotine-exposed mice than those from controls (p < 0.01, unpaired t-test).

(c) Cholinergic drive of fictive respiration in vitro
To evaluate the basal tonic cholinergic drive of fictive respiration, we studied the changes in amplitude and frequency induced by muscarinic (atropine) and nicotinic (hexamethonium) acetylcholine receptor blockers. In addition, we evaluated endogenous release of acetylcholine through the effects of neostigmine, an acetylcholinesterase blocker.

(i) Muscarinic and nicotinic receptor blockade
Atropine, but not hexamethonium, reduced the amplitude and frequency of basal (pH 7.4) fictive respiration
by approximately 30 per cent in en bloc preparations from control and nicotine-exposed mice ($p < 0.05$, Wilcoxon test). In contrast, neither atropine nor hexamethonium had a significant effect on fictive respiration recorded in slices from control or nicotine-exposed mice (figure 3a,b).

(ii) Acetylcholinesterase blockade
Superfusion with neostigmine reduced the amplitude and increased the frequency of basal (pH 7.4) fictive respiration ($p < 0.05$, Wilcoxon test, figure 3a,b) in en bloc preparations from control and nicotine-exposed mice. This dual effect of neostigmine is similar to that observed with administration of carbachol, a synthetic acetylcholine agonist (data not shown). In control slices, neostigmine also increased the frequency and decreased the amplitude. But, in slices from nicotine-exposed mice, neostigmine increased only the frequency of fictive respiration (figure 3b).
(d) Cholinergic contribution to central respiratory chemosensitivity

In order to evaluate the cholinergic contribution to central respiratory chemoreceptor mechanisms, changes in fictive respiration induced by acidification of the superfusion medium (from pH 7.4 to 7.1) were recorded in en bloc and brainstem slice preparations from controls and nicotine-exposed mice in the absence and presence of muscarinic (atropine 100 μM) and nicotinic (hexamethonium 100 μM) acetylcholine receptor blockers.

Essentially, the contribution of muscarinic acetylcholine receptors was diminished, while that of nAChRs was increased. In control en bloc preparations, atropine, but not hexamethonium, blocked the acidification-induced reductions in amplitude and increases in frequency of fictive respiration (figures 4a and 5a, p < 0.05, Wilcoxon test). In contrast, in en bloc preparations from nicotine-exposed mice, hexamethonium but not atropine blocked both the acidification-induced reductions in amplitude and the increases in frequency of fictive respiration (figures 4b and 5b).

In control slices, atropine or hexamethonium blocked the acidification-induced reduction in amplitude and the increase in frequency of fictive respiration (figures 4a and 5a, respectively, p < 0.05, Wilcoxon test). On the other hand, in slices from nicotine-exposed mice, no significant change in amplitude was observed in the absence or presence of atropine or hexamethonium. However, hexamethonium, but not atropine, blocked the acidification-induced increase in frequency observed in slice preparations from nicotine-exposed mice.

In summary, in general terms, in control preparations, atropine, but not hexamethonium, suppressed the respiratory responses to acidification of the superfusion medium from pH 7.4 to 7.1 (figures 4a and 5a). However, in preparations from nicotine-exposed mice, atropine did not block the chemosensory responses. In contrast, hexamethonium, which does not modify the chemosensory responses in control preparations, abolished the expected increase in respiratory frequency induced by acidification (figures 4a and 5a).

4. DISCUSSION

Our findings show that pre-natal nicotine exposure modifies the cholinergic contribution to central respiratory chemosensitivity. This switch from muscarinic to nicotinic receptor-based mechanisms is compatible with the known effect of pre-natal nicotine exposure of inducing downregulation of muscarinic acetylcholine receptors and upregulation of nAChRs in rat and mouse brains (Slotkin 1998). Pre-natal nicotine reduces the binding of M2 muscarinic receptors in the rat brainstem at early post-natal periods (Slotkin et al. 1999). In other systems, pre-natal nicotine can alter muscarinic receptor actions, either by uncoupling G-protein-dependent mechanisms in rat striatum and hippocampus (Zahalka et al. 1993) or by reducing mRNA of the muscarinic receptor in basal ganglia (Frank et al. 2001). Chronic nicotine exposure may lead to desensitization of nAChRs (Wang & Sun 2005) or their upregulation (Gaimarri et al. 2007). Chronic nicotine can affect selectively the number, stoichiometry, subunit composition and functionality of specific nAChRs (Van De Kamp & Collins 1994; Nguyen et al. 2003; Gaimarri et al. 2007; Walsh et al. 2008), especially the α4β2 subtype (Perry et al. 1999; Gentry & Lukas 2002).

Although en bloc and slice preparations differ in their responses in controls, both kinds of preparations showed modification of the cholinergic contribution to chemosensitivity after pre-natal nicotine exposure. The most obvious difference in the pattern of responses in controls is the magnitude of the reduction in amplitude induced by acidification. This is minimal in slices, despite the similar increases in frequency observed in both preparations. Differential distribution of cholinergic receptors along respiratory
brainstem nuclei may account for differential effects upon amplitude and frequency. Since the changes in frequency must rely on respiratory rhythm generators such as the pre-BötC, the changes in amplitude must be mostly related to other brainstem regions that are present in the en bloc preparation, but absent in slices. In agreement with this, neostigmine decreased the amplitude in en bloc but not in slice preparations.

Among the chemosensitive nuclei included in the slices, a possible target for nicotine actions is the pre-BötC. This chemosensitive nucleus (Solomon 2003) is crucial for generating the rhythm (Smith et al. 1991), and their neurons express M3 mAChR and α4β2 nAChR, receptors whose activation increases the respiratory frequency (Shao & Feldman 2002; Shao et al. 2008). Whether these receptor subtypes are involved in central chemosensitivity has not been defined. The greatest amount of mACHR binding in the respiratory network is found in the lateral and medial parabrachial nuclei and the lateral nucleus of the solitary tract (Mallios et al. 1995). Interestingly, mACHRs were also found in the nuclei of the VRG (nucleus ambiguus and retrofacial nucleus) and ventral medulla (retrotrapezoid nucleus and ventrolateral medulla), which could participate in the cholinergic drive of central chemosensitivity (Nattie et al. 1989; Guyenet et al. 2008). In addition, pre-natal nicotine exposure reduces α7 nAChRs in the forebrain and upregulates these in the brainstem and cerebellum of rats (Slotkin et al. 2004). It has been proposed that dysfunction of the α7 nAChRs can lead to impairment in the modulation of the pre-synaptic release of GABA and glutamate, which, in turn, could lead to changes in the density and/or function of post-synaptic receptors (Fregosi & Pilarski 2008). In fact, pre-natal nicotine exposure increases the inhibitory effects of GABA and glycine on pre-Bötzinger neurons (Luo et al. 2004, 2007).

Our results indicate that the tonic basal cholinergic drive of fictive respiration was not affected by pre-natal nicotine. Thus, the magnitude and pattern of changes in fictive respiration induced by neostigmine and acetylcholine receptor blockers were similar in control
and nicotine-exposed preparations. At a first glance, this result appears puzzling since the central chemo-
sensory response, which in part is mediated by cholinergic mechanisms, is decreased by pre-natal nicotine. Such disparity may reflect that cholinergic contribution to the central chemosensory is a low fraction of cholinergic mechanisms involved in respira-
tory neural control. In addition, our results show complex interactions between different cholinergic mechanisms. The prediction from neostigmine effects is that the cholinergic blockade during basal con-
tions should increase the amplitude and decrease the frequency of fictive respiration. Indeed, cholinergic blockade decreased both the amplitude and the fre-
quency of fictive respiration. Access of acetylcholine to extra-synaptic receptors might account for this discrep-
ancy. Whether adaptive mechanisms are triggered at the brainstem to counterbalance an increased
nicotinic contribution with a decay in the cholinergic
drive supported by muscarinic receptors is an open
question.

Finally, it should be remarked that nicotine infu-
sion alone is not a model for tobacco smoking, because nicotine is one of several chemicals in cigarette smoke that have addictive and potentially teratogenic effects (Rose 2006). In addition, mini-
pumps do not produce intermittent infusion of nicotine as occurs in smokers. However, they allow us to study the nicotine effects on brain development, mimicking the steady-state plasma levels of nicotine (15–45 ng ml⁻¹) observed in pregnant women con-
sidered moderate smokers (Benowitz & Jacob 1984)
but without confounding hypoxia and stress-derived factors associated with multiple subcutaneous injec-
tions. As well as exposure to tobacco smoke, nicotine infusion results in a similar upregulation of nAChRs
in the cortex and brainstem (Slotkin et al. 2002).

The nicotine infusion used in mice is 10 times greater
than that used in rats and produces reliable levels of
plasmatic nicotine around 250 ng ml⁻¹ (Robinson et al. 2002; Eugenin et al. 2008). Such doses induce
similar levels of nicotinic receptor upregulation in hypothalamus, hippocampus and cortex (Van De
Kamp & Collins 1994) and in respiratory-related regions of the brainstem in mice (Pauly et al. 1991;
Robinson et al. 2002). In addition, these doses do not affect litter size, birth weight or post-natal growth
curve in mice (Robinson et al. 2002; Eugenin et al. 2008).

In conclusion, we show that pre-natal nicotine exposure decreases the central chemosensory
responses and modifies the cholinergic contribution to central chemosensitivity, reducing muscarinic and
increasing nicotinic commands. Dysfunction in cen-
tral chemoreception and its cholinergic drive may play a role in disorders of respiratory control such as
SIDS. In fact, infant victims of SIDS show a decrease in the muscarinic binding in the arcuate
nucleus, which would contribute to the chemosens-
ory drive of respiration in humans (Kinney et al.
1995a,b).

Experiments were performed according to the Insti-
tute for Laboratory Animal Research (ILAR) Guide
for the Care and Use of Laboratory Animals and
approved by the Bioethics Committee of the Universidad de Santiago de Chile.

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