Antenatal insults modify newborn olfactory function by nitric oxide produced from neuronal nitric oxide synthase

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Newborn feeding, maternal, bonding, growth and wellbeing depend upon intact odor recognition in the early postnatal period. Antenatal stress may affect postnatal odor recognition. We investigated the exact role of a neurotransmitter, nitric oxide (NO), in newborn olfactory function. We hypothesized that olfactory neuron activity depended on NO generated by neuronal NO synthase (nNOS). Utilizing in vivo functional manganese enhanced MRI (MEMRI) in a rabbit model of cerebral palsy we had shown previously that in utero hypoxia-ischemia (H–I) at E22 (70% gestation) resulted in impaired postnatal response to odorants and poor feeding. With the same antenatal insult, we manipulated NO levels in the olfactory neuron in postnatal day 1 (P1) kits by administration of intranasal NO donors or a highly selective nNOS inhibitor. Olfactory function was quantitatively measured by the response to amyl acetate stimulation by MEMRI. The relevance of nNOS to normal olfactory development was confirmed by the increase of nNOS gene expression from fetal ages to P1 in olfactory epithelium and bulbs. In control kits, nNOS inhibition decreased NO production in the olfactory system and increased MEMRI slope enhancement. In H–I kits the MEMRI slope did not increase, implicating modification of endogenous NO-mediated olfactory function by the antenatal insult. NO donors as a source of exogenous NO did not significantly change function in either group. In conclusion, olfactory epithelium nNOS in newborn rabbits probably modulates olfactory signal transduction. Antenatal H–I injury remote from delivery may affect early functional development of the olfactory system by decreasing NO-dependent signal transduction.

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Introduction

It is increasingly being recognized that an enriched environment is needed for developing the sensory capabilities and preferences of newborns (Schaal et al., 2004). Olfactory cues soothe newborns, and olfactory familiarity with the mother may help the newborn transition to extra-uterine life (Varendi et al., 1997; Winberg and Porter, 1998) and promote early mother–infant attachment (Varendi et al., 1997; Winberg and Porter, 1998). Breast feeding, depends on olfactory cues (Varendi et al., 1994), and the odor of breast milk increases non-nutritive sucking in tube fed premature infants (Bingham et al., 2007). Early olfactory conditioning contributes to the development of preferences in the alimentary (including obesity) and social domains (Varendi et al., 1994; Winberg and Porter, 1998) and has a developmentally sensitive period (Wilson and Sullivan, 1994). Improvements in neonatal care have increased premature newborn survival but the routine neonatal intensive care practices of nasal cannula and other respiratory devices, and early feeding with oro- or nasogastric gavage result in a chemo-sensory experience that is detrimental to normal development (Kuhn et al., 2011). In the rabbit model of cerebral palsy (CP) (Derrick et al., 2007), the motor responses of kits to odorants and MRI measures of olfactory function are impaired (Drobyshevsky et al., 2006) implicating the importance of antenatal stress in the development of abnormal olfactory function.

Nitric oxide (NO) is assumed to play a key role in the development (Bredt and Snyder, 1994) and functioning (Breer and Shepherd, 1993; Lowe et al., 2008; Wilson et al., 2007) of the olfactory system. The neuronal nitric oxide synthase isofrom (nNOS) is transiently overexpressed late in gestation and early postnatal period in the rodent
olfactory epithelium (OE) and remains high in olfactory bulbs (OB) through adulthood (Bredt and Snyder, 1994; Chen et al., 2004). Nitric oxide mediates formation of synaptic connections in developing and regenerating olfactory receptor neurons (ORN) (Roskams et al., 1994) and is essential in shaping glomerular organization in the OB (Chen et al., 2004).

The exact function of nNOS in the peripheral olfactory system remains controversial (Wilson et al., 2007), especially whether nNOS participates in olfactory signal transduction in the OE, as previously suggested (Breer and Shepherd, 1993). Previous studies utilized NOS inhibitors with low nNOS specificity (Romero-Grimaldi et al., 2008), which resulted in some doubt about the exact contribution of the isoform nNOS. New selective nNOS inhibitors now allow us to investigate specific involvement of endogenous nitric oxide in the function of a single central nervous system (CNS) neuron. We hypothesized, that during the perinatal period in which there is a high expression of nNOS in the OE, ORN activity depends on NO generated by nNOS in response to olfactory stimuli. We further hypothesized that NO-regulated pathways are involved in postnatal sensory deficits following antenatal insults.

Maternal administration of a selective nNOS inhibitor prior to antenatal hypoxia–ischemia (H–I) has a neuroprotective effect on postnatal motor deficits in rabbits (Yu et al., 2011). Using a highly specific novel nNOS inhibitor and the ORN response to olfactory stimulation with functional manganese enhanced MRI (MEMRI), we show here that antenatal H–I injury at premature gestation affects NO-regulated pathways in olfactory signal transduction at term in postnatal rabbit kits.

Methods

The Institutional Animal Care and Use Committee of NorthShore University HealthSystem Research Institute approved all experimental procedures with animals.

Antenatal hypoxia–ischemia

The surgical procedure has been previously described (Derrick et al., 2004). In vivo global H–I of fetuses was induced by sustained 40-min uterine ischemia at 22 days gestation (70% term) in timed pregnant New Zealand white rabbits (Myrtle’s Rabbitts, Thompson Station, TN). This procedure models acute placental insufficiency at a premature gestation. Briefly, dams were anesthetized with intravenous fentanyl (75 μg/kg/h) and droperidol (3.75 mg/kg/h), followed by spinal anesthesia using 0.75% bupivicaine. A balloon catheter was introduced into the left femoral artery and advanced into the descending aorta to above the uterine and below the renal arteries. The balloon was inflated for 40 min causing uterine ischemia and subsequent global fetal H–I. At the end of H–I, the balloon was deflated, resulting in uterine reperfusion and reperfusion–reoxygenation to the fetal brains. The catheter was removed, the femoral artery reconstructed, and the dam returned to her cage and allowed to deliver spontaneously.

On postnatal day 1 (P1) kits underwent neurobehavioral testing for the presence of sensory and motor deficits, including the test for aversive reaction to amyl acetate as described previously (Derrick et al., 2004). Olfaction was tested by aversive response to a cotton swab soaked with odorant. The test was videotaped, and the results were scored from 0 (no reaction) to 4 (active avoidance), assessed by 2 observers who were blind to the experimental groups. In our previous study (Drobyshevsky et al., 2006) MEMRI found deficits in olfactory function that were not detected by neurobehavioral tests. In the current study, we expanded the olfactory scoring range from 0 to 4 for the neurobehavioral tests.

The surviving kits were categorized as 1) non-hypertonic kits with normal tone in all limbs and 2) hypertonic kits with increased muscle tone. Hypertonia was defined as increased resistance to passive stretch in any limb. Immediately after neurobehavioral assessment the newborn P1 kits underwent MEMRI examination.

Experimental groups

Two groups of P1 kits were examined in the study: 1) kits after global antenatal 40 min H–I at E22. Only kits with muscle hypertonia and motor deficits were included in the H–I group of the study. The presence of hypertonia was used as an indication of significant CNS brain injury in newborn kits. 2) Naïve newborn P1 kits served as a control group. In our experience, even with 30 min hypoxia–ischemia at E22, the postnatal kits at P1 were indistinguishable from naïve controls in the motor response to olfactory function. E22 fetuses well tolerate sham surgery and newborn rabbit kits born 10 days are exactly like naïve controls. Since our goal was to also to delineate carefully the normal behavior, we chose to use naïve control kits.

The drug subgroups were as follows:

- NO donors: Spermine NONOate (Sigma, St. Louis, MO) or DETA NONOate (Sigma, St. Louis, MO), were administered intranasally in a dose of 25 μl/nostril of 1000 μM solution.
- To control for the effect of off-target effects, we elected to use inactive NO donors (instead of vehicle) to serve as controls for drug subgroup 1 above. NO donors were made inactive by keeping them at room temperature and exposed to light for 24 h. These controls were better than saline as the vehicle, as they would have only one difference from the drug, which was the absence of donation of NO.
- nNOS inhibitor: JI-5 (cis-N1-[4′-6′-amino-4′-methyl-pyridin-2′-ylmethyl]-pyrrolidin-3′-yl)-[N2-2′-[3′fluoro-phenyl]-ethyl]-ethane-1, 2-diamine) in two doses was used. JI-5 is a highly selective nNOS inhibitor with Ki for nNOS of 0.0053 μM (Delker et al., 2010). The Ki of JI-5 for nNOS is 743 and 3830 times less than iNOS and eNOS respectively. JI-5 was administered intranasally 0.025 mL/nostril of either 1.59 μM or 3.08 μM. These concentrations were estimated to reach 300 Ki and 600 Ki, respectively, in the olfactory neuron, if there was perfect distribution across the entire kit. We have shown that the new class of nNOS inhibitors is far superior to 7-nitroindazole, the previous favorite nNOS inhibitor, in neuroprotection for behavioral endpoints as well as side effects (Yu et al., 2011).
- Control for the nNOS inhibitors (subgroup 3) was equi-volume administration of the saline vehicle.

Time of administration

Timeline of MEMRI experiments is presented in Fig. 1. NO donors and JI-5 were administered 60 min before imaging. We were targeting the effect of NO produced by nNOS only for 60 to 150 min on olfactory function. The JI-5 group would thus have an absence of NO production for 60 to 150 min but would not necessarily get rid of the NO present which was produced before Time 0.

Manganese enhanced MRI imaging

Uptake of paramagnetic Mn2+ ion via Ca2+ voltage gate channels of olfactory neurons can be registered on T1-weighted images. Signal enhancement serves as a surrogate marker for olfactory neuronal activity (Pautler et al., 1998). We have previously shown that the dynamic imaging of a T1-weighted signal in OB reveals information about the rate of Mn2+ ion uptake, recorded as the slope of signal enhancement, as well as the speed of axonal transport in olfactory neurons, recorded as the onset of signal enhancement (Drobyshevsky et al., 2006).

Manganese chloride was obtained from Sigma (St. Louis, MO) and dissolved in saline. Kits were administered MnCl2 intranasally by pipette 90 min before imaging in a dose of 10 mg/kg in 0.02 mL per
nostril. The animals were placed in a chamber and were given odor stimulation by blowing 2 L/min air passing through a test tube containing 1 mL of amyyl acetate (Sigma, St. Louis, MO), diluted 1:10 in mineral oil. Stimulation consisted of blocks of odorant (30 s) or clear air (2 min) alternately for 15 min.

One hour after manganese chloride administration, rabbit kits were sedated with intramuscular injection of ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (1.0 mg/kg). Imaging was performed in 4.7 T Bruker BioSpec scanner with 93 mm birdcage coil. Series of T1-weighted spin-echo images (RARE-T1 sequence, RARE factor 4, TR/TE = 842/10 ms, NEX = 3, FOV 70 mm, matrix 256 × 128, slice thickness 1 mm, 1 min volume acquisition time, 150 volumes for 2 h 30 min) were acquired. Simultaneous imaging of 8 kits by a custom made animal holder allowed us to remove biases in imaging at different times, scanner drift, and temperature variation and prevents different drug exposure times. To control for inter-experiment variables, kits were randomly assigned to a drug or corresponding control in each imaging session. Inter-session controls were obtained to account for scanner drift, change of body temperature and other possible factors. The gradient cooling system settings were adjusted to keep ambient air temperature inside the magnet bore at 28 °C and kits’ body core temperature at ~35 °C. Body core temperature was monitored in one of the kits during an imaging session using an MRI compatible fiber optic probe (FOTS100, Biopac Inc., CA).

Rabbit kits were positioned in the cradle in groups with an equal number of randomly assigned kits, facing each other toward the magnet isocenter. Eight coronal slices were placed in two slice groups (16 slices total) covering the head region from the nasal turbinates and OE to the beginning of the piriform cortex. Regions of interest (ROI) were placed on the glomerular layer of OB (Fig. 2). T1-weighted images were normalized by values from the ROI placed on a kit’s eye in a corresponding slice, where there is no enhancement by Mn²⁺. Onset and slope of signal enhancement in the OB, corresponding to arrival and accumulation of Mn²⁺ were determined from the ROI signal time course as before (Drobyshevsky et al., 2006) as outcome parameters.

To account for a possible difference in kits’ sizes between litters, changes in ambient and body temperature (Smith et al., 2007), scanner drift, olfactory exposure and other intersession experimental confounds, two levels of normalization procedures were applied. First, a signal from OB was normalized to a region of interest in the kit’s eye, having the same temperature, but no manganese uptake. Second, kits from the same litter were randomly divided into saline and drug groups, and imaged simultaneously using the custom made animal holder. MRI derived parameters in the drug groups were normalized to the average values of control group littersmates of the same day.

Determination of nitrogen oxides (NOx)

NO production in the OE and OB was indirectly determined by measuring nitrogen oxides (NOx) consisting of NO₂ and NO₃ (Michelakis and Archer, 1998). The effect of nNOS inhibition on NO production was monitored starting 60 min prior to imaging. Immediately after MRI (4 h after intranasal application of NOs inhibitors), samples of OE and OB were obtained. Fresh tissue was frozen in liquid nitrogen and then stored at −80 °C. For analysis, the tissue was defrosted and homogenized with an ultrasonic homogenizer. The homogenate was centrifuged at 4200 rpm for 15 min at 4 °C. The supernatant was kept on ice and used immediately for analysis. NOx was detected with a Nitric Oxide Chemiluminescent Analyzer, Sievers 280i NOA™ (Sievers Instruments, Boulder, CO). To measure nitrate, vanadium (III) chloride in hydrochloric acid at 90 °C was used to convert nitrate to NO. To measure nitrite, sodium iodide in acetic acid converted nitrite to NO, and NO was detected by chemiluminescence. Samples were compared to a range of standards of sodium nitrate and nitrite. The difference of NOx measurements between nNOS inhibitors and vehicle controls gave an indirect estimate of the actual NO produced due to nNOS.

Gene expression

Gene expression of nNOS was determined in the OE and olfactory bulb at various gestational ages. Total RNA from different brain regions and gestational age fetuses (n = 4/age) was extracted with RNeasy Protect Cell Mini Kit (Valencia, CA) and 2 μg of RNA was reverse transcribed to cDNA with High Capacity cDNA Reverse Transcription Kit (ABI, Foster City, CA). SYBR Green based real-time PCR primers were designed based on the full sequence of rabbit nNOS cDNA and rabbit 18s RNA with Primer Express (ABI, Foster City, CA). Real-time PCR was conducted with SYBR Green Master mix, the signal collected by Rotor Gene system with 1 μl of RT reaction loaded into each 20 μl real time PCR reaction. The relative steady state levels of mRNA were calculated with ΔΔCT and rabbit 18s as an internal standard.
control. For rabbit nNOS: forward, 5′-CAG AGA CCA CTT TGA GAG CGC; reverse, 5′-ACG GAG AAC CTC ACA TTG GC.

Nasal epithelium blood flow

Micro-vascular blood flow change in OE was measured with a laser Doppler flow meter (PeriFlux System 5000, Perimed Inc., OH) and intranasal microprobe (probe 415–230). Relative blood flow in arbitrary units, calibrated to motility standard, was recorded from the anesthetized rabbit kit before and after intranasal administration of 50 μL saline and 100 μM of NaNO2, and NaNO3.

Statistical analysis

Data is presented as means ± SEM. Comparisons across groups were done with a t-test, ANOVA, or Wilcoxon signed rank test where appropriate. Differences with p-values less than 0.05 were considered significant. Correlations between ordinal neurobehavioral scores of smell with MEMRI were done with the Spearman correlation coefficient (rS). Developmental changes in gene expression were tested using repeated measures ANOVA.

Results

Developmental profile of NOS gene expression in olfactory bulbs

Olfactory bulb nNOS gene expression increased rapidly from late fetal to early postnatal period (Fig. 3A). In contrast the other 2 isoforms of NOS (iNOS and eNOS) remained unchanged. Newborn rabbit kits do not respond to sound or light stimulation at P1 which makes olfactory stimulation by ambient odors, including that of the mother, mother’s milk and the nest important. The seven fold increase of nNOS in newborn rabbit kits likely indicates the importance of nNOS in smell recognition and learning after delivery. Gene expression of nNOS did not significantly change in OB of H–I rabbit kits at E29 (0.28 ± 0.08 vs 0.03 ± 0.01, NS), and P1 (0.33 ± 0.05 vs 0.51 ± 0.08, NS), as well as in OE at E29 (0.06 ± 0.02 vs 0.01 ± 0.00, NS), and P1 (0.44 ± 0.21 vs 0.04 ± 0.01, NS), compared to controls (data normalized to OB adult nNOS level).

Gene expression of nNOS in the OE was significantly upregulated in newborn kits at P1–P2 and gradually decreased with maturation (Fig. 3B). Gene expression of nNOS in OB progressively increased from the fetal through the postnatal period and peaked around the 3rd postnatal week and then decreased and remained stable till adulthood (Fig. 3B).

Suppression of nNOS activity in the olfactory system by selective inhibitors

To examine the role of NO in perinatal smell function development we manipulated NO levels by either blocking nNOS activity with JI-5, a highly selective inhibitor of nNOS, or increasing NO levels with NO donors. NO production was determined by measuring NOx in tissues 4 h after intranasal application of equal volumes of either saline or nNOS inhibitor. NO production in the saline group was significantly higher in OB, 6.01 ± 0.59 mmol/mg protein, than in the nasal OE, 4.85 ± 0.45 mmol/mg protein. To account for possible differences in litter sizes and kit weights, ambient temperature, olfactory exposure and other inter session experimental confounds, NOx tissue concentrations in OE or OB were normalized by the average NOx concentration in corresponding tissue from the same day saline group littermates. Intranasal application of nNOS inhibitor significantly decreased NO production in OE and OB (Fig. 4). Relative suppression of NO production by nNOS inhibitor JI-5 was similar in OE (82.4%) and in OB (82.5%).

Inhibition of nNOS increases olfactory neuronal activity

Next we evaluated whether decreasing NO in the olfactory system affects olfactory neuronal activity in response to odor stimulation using manganese enhanced MRI.

JI-5, the nNOS inhibitor, was administered in the newborn kits’ nostrils 1 h prior to contrast administration and odor exposure. The slope of signal enhancement in control kits was significantly increased when the high dose of JI-5 (600 Ki) was applied (Fig. 5A). The slope of signal enhancement in H–I kits was not significantly increased when either dose of JI-5 was applied (Fig. 5A). There was no difference in the other parameter of MEMRI, onset of enhancement, between saline and JI-5 in control or H–I groups (Fig. 5B).

Supplementation of NO did not affect olfactory neuronal activity

The NO donor DETA NONOate, applied intranasally 1 h prior to contrast administration and odor stimulation did not significantly change the slope of signal enhancement (normalized to average inactive form, 1.00 ± 0.30 drug vs. 1.00 ± 0.27 inactive form, NS) either in the contrast arrival time (1.00 ± 0.02 drug vs. 0.90 ± 0.02 inactive form, NS), in control P1 kits. We suspected that there was not enough incubation time for the compound to release sufficient NO, since the
peak of NO production of this compound is 4–6 h. For this reason the next experiment used the faster releasing NO donor, Spermine NONOate.

The NO donor Spermine NONOate, applied intranasally 1 h prior to contrast administration and odor stimulation also did not significantly change the slope of signal enhancement either in controls or in the H-I group compared to the group that received the inactive form (Fig. 6A). There was also no difference between groups in the contrast arrival time, suggesting that the speed of axonal transport in OE neurons is not affected by exogenous NO in both control and H-I groups (Fig. 6B).

Slope of contrast enhancement correlated with olfactory scores in control group

No significant correlation between MEMRI parameters and smell score was observed in vehicle control groups ($r_s = 0.31, p = 0.66$ for enhancement slope in expired Spermine NONOate group). However, with Spermine NONOate application, control kits with higher smell scores had larger enhancement in slope (Fig. 7). The correlation between olfactory scores and slope of enhancement was significantly different from zero, $r_s = 0.57, p = 0.020$. There were no significant correlations between MEMRI parameters and behavioral olfactory scores with JI-5 administration.

Effect of nitrites and nitrates on nasal epithelium blood flow

We confirmed that changes in olfactory receptor neuron responses on MEMRI could not be attributed to epithelial blood flow changes caused by metabolites of NO, since we did not observe significant differences in microvascular blood flow change in OE between saline and NO oxidation products ($\text{NaNO}_2$ or $\text{NaNO}_3$, 100 μM, $n = 3$ kits). Microvascular blood flow was measured by laser Doppler flow meter and intranasal microprobe in anesthetized P1 rabbit kits. After a short spike in response to liquid administration, blood flow returned to baseline within a few minutes (Fig. 8A). The experiment demonstrated that there was no long term effect of intranasal nitrite and nitrate on microvascular blood flow (Fig. 8B).

Discussion

Using genetic and functional methods, we provide evidence that nNOS is present in OE of a newborn rabbit and modulates olfactory signal transduction. This function is altered by antenatal H-I injury. In control kits nNOS inhibition decreased NO production in the olfactory system and significantly increased MEMRI slope enhancement. In H-I kits the MEMRI slope did not increase with decreased NO. NO donors did not significantly change MEMRI slope or onset in either group. However, the correlation of MEMRI slope with behavioral score was affected in control but not in H-I kits. This reinforces the view of modification of endogenous NO-mediated olfactory function by the antenatal insult.

Previous studies have demonstrated that the role that nitric oxide plays depends upon its site of production, as nNOS expression is dynamic during development and during the postnatal period (Roskams et al., 1994). In developing ORNs, nNOS is highly expressed during embryogenesis (Roskams et al., 1994), similar to its expression in the developing cortex (Bredt and Snyder, 1994), but is downregulated shortly after birth (Roskams et al., 1994). Using nNOS-deficient mice it has been shown that the role of nNOS in development depends on its localization: it promotes neurogenesis in early postnatal development in OE and glomerular organization in the OB in adult (Chen et al., 2004). The dramatic decrease in proliferation of neuronal precursors seen in nNOS null mice at the first postnatal week can be explained if nNOS acts as an autocrine or paracrine neuroproliferative factor. While the pool of neuronal precursors and neurogenesis normalized in the nNOS
null mouse by sixth postnatal week, there was an overgrowth of mitral or tufted cell dendrites and a decreased number of active synapses in the OB. Mice deficient in nNOS also have decreased levels of cyclic 3′,5′-guanosine monophosphate (cGMP), suggesting that nNOS activity plays a role in olfactory system organization. Further evidence for the function of nNOS in OE as a modulator of neurogenesis comes from studies of OB removal, which results in retrograde neuronal degeneration and repopulation of the epithelium (Weruaga et al., 2000) and is associated with up-regulation of nNOS expression. nNOS has been assumed to play a role in proliferation of ORNs but not in olfactory signal transduction as nNOS is downregulated by the second week of postnatal development and is absent from ORNs in adult rodents (Bredt and Snyder, 1994; Roskams et al., 1994).

Based on NADPH diaphorase staining in ORNs and a NO-sensitive guanyl-cyclase, it has been proposed that NO may have several functions in ORN signal transduction (Breer and Shepherd, 1993). First, NO, can easily diffuse across cell membranes and may recruit adjacent ORNs to encode very intense stimuli by the cGMP pathway. At the same time such activation may promote functional and structural organizations of ORN clusters responding to similar odorants (Moulton, 1976). Second, slow elevation of cGMP may play a role in sensory adaptation of ORNs to prolonged and intense stimuli.

High levels of NO and cGMP are found in the OB (Lowe et al., 2008). There is an increase of NO during olfactory stimulation (Lowe et al., 2008). nNOS is highly expressed in the periglomerular and granule cells in the OB in neonates and adults (Bredt and Snyder, 1994; Chen et al., 2004; Roskams et al., 1994).

There has yet been no conclusive evidence that nNOS is a component of normal signal transduction in the ORN (Roskams et al., 1994). Some recent experimental data provide evidence that eNOS is present in adult OE (although not in ORN) (Brunert et al., 2009). Using NO-sensitive microelectrodes, they showed that stimulation liberates NO from isolated wild-type ORNs, but not from ORNs of eNOS deficient mice. Integrated electrophysiological recordings from the OE of these mice show that NO facilitates adaptation for repetitive olfactory stimuli. However, the functional role of nNOS in stimulus dependent activity maturation during early postnatal development has not been previously recognized.

This study provides first time experimental evidence in support of the role of nNOS in normal olfactory signal transduction during the early postnatal period. Quantitative gene expression analysis revealed that, in rabbits, nNOS expression remains relatively high for at least three postnatal weeks (Fig. 3B), similar to those in rodents (Bredt and Snyder, 1994; Roskams et al., 1994), with the exception that epithelial nNOS did not completely disappear in mature rabbits. This suggests that nNOS does more than just regulate neurogenesis in the perinatal period. Using highly selective nNOS inhibitors we demonstrated that formation of NO due to nNOS activity is present in P1 rabbit kits, both in OE and OB (Fig. 4), and production in 4 h was equivalent to about 20% of cumulative NO production from all NOS isoforms at all proximate sites. Most importantly, we showed that ORN activity in response to olfactory stimulation is modulated by nNOS activity (Fig. 5). In fact, ORN activity was increased when NO production was decreased with application of nNOS inhibitors and did not change with application of NO donors. This effect could not be attributed to the prolonged increase of OE blood flow, as indicated by microvascular blood flow response recordings (Fig. 8). The absence of an effect of NO donors on ORN activity, despite possible supra-physiological NO levels, emphasizes the importance of localized NO production by nNOS in ORNs in response to olfactory stimulation. It is possible that NO behaves in a threshold stimulatory manner, and exogenous NO does not enhance the inhibitory effect on olfactory function.
Currently it is unclear what causes the increase of ORN activity after inhibition of NO production. We hypothesize that it may be related to the decrease of lateral inhibition by NO (Breer and Shepherd, 1993) and therefore decreased adaptation to the repetitive olfactory stimulus. In the current study, olfactory stimulation consisted of long blocks of odorant (30 s) intermittent with clear air (2 min) alternately for 15 min. This stimulation protocol, typical for olfactory MEMRI studies (Pautler and Koretsky, 2002), is designed to increase total uptake of saline or sodium nitrite at 21 °C (B). The order of saline or sodium nitrite was switched in the left and right nostrils of the same animal and the effect was the same. The difference in the blood flow response magnitude between saline and sodium nitrite was not significant. The power of the t-test is 0.17. Ruling out a Type II error with power = 0.80 would require 29 animals in each group.

In hypoxic human infants, inhaled nitric oxide (NO) given as a gas is currently used as therapy for modulation of pulmonary vasculature (Barrington and Finer, 2006). One clinical study (Marks and Schreiber, 2008) and some animal studies (Olivier et al., 2010; Pansiot et al., 2010) have suggested that inhaled nitric oxide may also have a neuroprotective effect, although this has not been borne out in subsequent clinical studies in premature infants (Cole et al., 2011; Mercier et al., 2010). Although the current study used short and reversible NO modulation in the olfactory system, the emerging role of peripheral olfactory nNOS in the functional shaping of the adult olfactory system warrants further study of prolonged NO modulation in the perinatal period.

As a conclusion, the results of the study indicate that nNOS is present in rabbit OE during the first three postnatal weeks. Modulation of olfactory transduction by NO levels suggests a new role of OE nNOS in activity dependent functional maturation of the olfactory system during the perinatal period. Perinatal H-I injury may affect early functional development of olfaction by decreasing NO-dependence of olfactory signal transduction.

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