Abnormal Immunoreactivity to Serotonin in Cerebellar Purkinje Cells after Neonatal Cocaine Exposure

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ABSTRACT: **Neonatal cocaine is known to affect the developing serotonergic system in many brain structures, including the cerebellum. Changes in the cerebellar Purkinje cells after drug exposure are well documented and result in impairment of movement and other cerebellar disorders such as ataxia. These cells have a major postnatal developmental pattern; therefore, neonatal exposure to cocaine is likely to affect them. In this work, male and female Wistar rats were injected with 15 mg of cocaine hydrochloride/kg body weight/day, subcutaneously, in two daily doses, from postnatal day 1 (PND1) to PND29. Controls were given 0.9% of saline. On PND14, PND21, and PND30, rats were transcardially perfused, and brains removed and cryoprotected. Coronal sections from the cerebellum were processed for immunocytochemistry of cells containing serotonin (5-hydroxytryptamine, or 5-HT). At the same postnatal age, rats from at least three different litters were sacrificed by decapitation, and brains were dissected for determination of 5-HT in the cerebellum by high-performance liquid chromatography with electrochemical detection. Upon the expected distribution of immunoreactivity to 5-HT, an abnormal immunoreactivity to 5-HT was observed in the Purkinje cells of six cocaine-exposed animals, but not in control animals. Also, levels of cerebellar 5-HT in cocaine-exposed rats were significantly increased on PND21. These results, together with previously reported observations of altered patterns of motor behavior, indicate that neonatal cocaine exposure affects the serotonergic cerebellar system, altering the standard development of Purkinje cells and possibly compromising the motor function.**

KEYWORDS: **cocaine; neonatal; serotonin (5-hydroxytryptamine, or 5-HT); cerebellum; Purkinje cells; rat**

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Ann. N.Y. Acad. Sci. 1025: 630–637 (2004).
doi: 10.1196/annals.1316.078

INTRODUCTION

Many studies have shown detrimental action of cocaine in different regions of the developing brain.[1–3] However, only a few studies have examined the effects of cocaine exposure in the cerebellum.[4–7] Nevertheless, it was suggested that following exposure to drugs of abuse, the cerebellum may be responsible for mood changes, a different role than its classical action in motor control.[8] Also, cocaine exposure was shown to decrease Purkinje cell spontaneous and glutamate-evoked firing[7] or γ-aminobutyric acid (GABA)-induced discharges,[4] suggesting that by changing the normal function of the cerebellum, cocaine can produce drug-related alterations in behavior and cognition.[4,7]

In the rat, cerebellar Purkinje cells are generated from embryonic day 13 to embryonic day 15, but present a major postnatal developmental pattern because their dendritic trees develop from birth until the third postnatal week.[9] Therefore, neonatal exposure to cocaine is likely to affect the development of these cells. Depression of Purkinje cells by ethanol exposure is well documented, causing dendritic regression and disruption of the cortical structure,[10–17] and resulting in impairment of movement and other cerebellar disorders such as ataxia.[11,18–20]

The cerebellum is well known to be involved in motor regulation.[21,22] The level of serotonin (5-hydroxytryptamine, or 5-HT) in the cerebellar cortex was shown to be related to the level of motor activity.[23] The cerebellar cortex was also reported to play a role in controlling motivation to explore a novel environment.[24] This finding strengthens the hypothesis that the cerebellum is implicated in motivational processes.

We report here effects of rat neonatal cocaine exposure in the cerebellar serotonergic system, throughout the first month of life, consistent with the behavioral alterations we have previously reported.[25,26]

MATERIALS AND METHODS

Wistar rat litters were culled to eight pups each, with equivalent sex representation, on postnatal day 1 (PND1). Animals were injected subcutaneously from PND1 to PND29 with 15 mg/kg of body weight/day of cocaine hydrochloride (Sigma, St. Louis, MO) in two daily doses (at 9:00 AM and 5:00 PM), or they were injected with an isovolumetric dose of saline vehicle. Only two male and two female rats from each litter were used for a given study. On the day of sacrifice (PND14, PND21, or PND30), rats were not injected.

On PND14, PND21, and PND30, pups used for neurohistological determinations were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4). Brain coronal sections of 40 μm were obtained on a freezing microtome (CM1325, Leica Microsystems, Heidelberg, Germany). Immunoreactivity to 5-HT was carried in free-floating sections using a TR-125-HL kit (Lab Vision, Fremont, CA). Sections were first incubated in 0.1% hydrogen peroxide to reduce endogenous peroxidase activity. Sections were subsequently incubated in Ultra V Block (from the TE-125-HL kit) to reduce nonspecific background staining, and then they were incubated for 36–48 h (at 4°C with continuous shaking) with an antibody to 5-HT (kindly offered by Professor John Parnavelas, University Col-

lege London, London, U.K.), used at a titer of 1:10000 in phosphate-buffered saline. Visualization of 5-HT-positive cells was made using streptavidin peroxidase and 3,3′–diaminobenzidine as a chromogen. The specificity of the immunoreactivity to the primary antibody was tested in different ways, including omitting the primary antibody itself, or the secondary antibody from the experimental procedure. Omission of the 5-HT antibody or secondary antibody eliminated all immunostaining. Sections of the control group were always processed in parallel with sections from the cocaine-exposed group. Each experimental group consisted of five to six animals from at least three different litters.

Animals used for neurochemical determinations were sacrificed by decapitation on PND14, PND21, or PND30. Brains were rapidly removed, and dissection of the cerebellum was performed on ice. Tissue samples were frozen by immersion in 4-methylbutane over dry ice and stored at –70°C until used for neurochemical determinations. Levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were quantified by a modified method[27] of high-performance liquid chromatography combined with electrochemical detection (Gilson, Middleton, WI). The analytic column was a Supelco Supelcosil LC-18 3 μM (7.5 cm × 4.6 mm). Concentrations of 5-HT and 5-HIAA were calculated using standard curves. Standards of 5-HT and 5-HIAA were purchased from Sigma. Final results were expressed in terms of monoamine content per amount of protein. The total content of protein was assayed in duplicate using a colorimetric microassay from Bio-Rad based on the Bradford assay.[28] A minimum of six animals (from at least three different litters) were used per experimental group.

Neurochemical data were initially analyzed using a three-way multivariate analysis of variance (treatment × age × gender). As no effect or interaction of gender was detected, data were collapsed across gender and reanalyzed by a two-way analysis of variance (treatment × age). Significant main effects and interactions were further explored using *post hoc* contrasts. Planned comparisons with contrast analysis were conducted to investigate all possible meaningful significant differences. The statistical level of significance was considered to be at $P < .05$.

RESULTS

We have noticed an abnormal immunoreactivity of the cerebellar Purkinje cells against the antibody anti-5-HT (FIG. 1A and C) with respect to the expected distribution of immunoreactivity to 5-HT (FIG. 1B).[29] This pattern of immunoreactivity was observed in 6 out of 30 cocaine-exposed animals, which represents a significant number of animals with abnormal immunoreactivity to 5-HT ($P < .01$). This immunoreactivity was detected in the cerebellar sections of two animals on PND14, one animal on PND21, and three animals on PND30, all of them neonatally exposed to cocaine. No immunoreactivity to the antibody anti-5-HT was observed in the Purkinje cells from sections obtained from control animals (FIG. 1D). It is interesting to notice that the number of Purkinje cells marked against 5-HT was not the same in all animals. Interestingly, the animal that presented the higher number of marked Purkinje cells was also found to display immunoreactivity to the antibody we have used against tyrosine hydroxylase in these cells (data not shown).

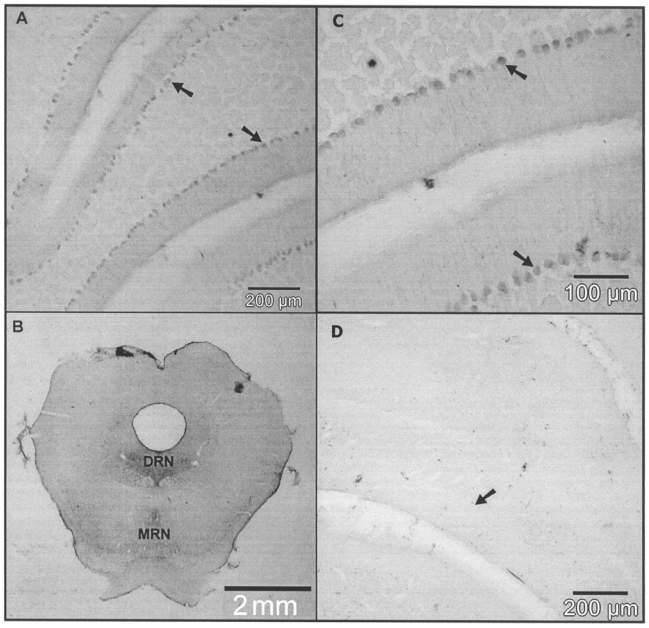

FIGURE 1. (**A**) Cerebellar coronal section from a cocaine-exposed rat, showing Purkinje cells marked by the antibody used against 5-HT–positive neurons (note the scale bar in the lower right corner). (**B**) Typical aspect of the immunoreativity against 5-HT, at the level of the dorsal raphe nuclei (DRN) and the medial raphe nuclei (MRN), illustrating the specificity of the antibody used. (**C**) Detail of the section shown in (**A**) (note the scale bar in the lower right corner). (**D**) Cerebellar coronal section of a saline-injected animal (note the scale bar in the lower right corner). *Black arrows* indicate Purkinje cells.

Levels of 5-HT and 5-HIAA in the cerebellum, analyzed by a two-way analysis of vairance, revealed the main effects of treatment [$R(2,83) = 5.26$, $P < .01$] and age [$R(4,166) = 27.55$, $P < .00001$], and an interaction between treatment and age [$R(4,166) = 9.61$, $P < .00001$]. Further testing revealed that pups neonatally exposed to cocaine presented increased levels of 5-HIAA on PND14 [$F(1,84) = 4.60$, $P < .05$] and increased levels of 5-HT and 5-HIAA on PND21 {5-HT: [$F(1,84) = 42.39$, $P < .00001$]; 5-HIAA: [$F(1,84) = 14.13$, $P < .001$]} when compared to control animals. On PND30, pups exposed to cocaine also presented increased levels of 5-HIAA [$F(1,84) = 4.34$, $P < .05$]. Levels of 5-HT and its metabolite, obtained by contrast analysis, are graphically represented in FIGURE 2. The levels are expressed as ng of indolamine/mg of protein, and each result represents a mean ± SEM. Significant differences between groups are also indicated.

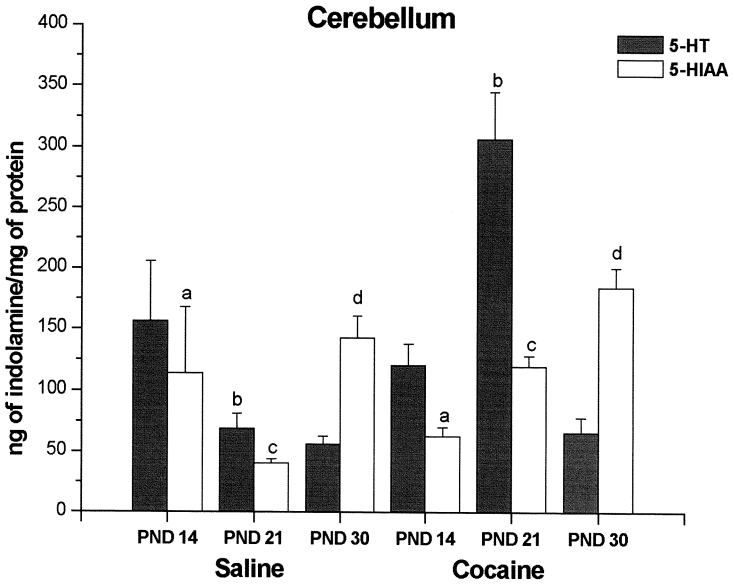

FIGURE 2. Effects of neonatal cocaine exposure in the cerebellar concentrations of 5-HT and its metabolite 5-HIAA determined by high-performance liquid chromatography with electrochemical detection. Each column represents a mean ± SEM from 12 to 14 animals per group. Columns with the same letter are significantly different from each other: a and d: $P < .05$; b and c: $P < .001$.

DISCUSSION

Throughout development, 5-HT is known to play a trophic role in the differentiation of neural cells, participating in the assembly of the central nervous system from its origin through the terminal events of axonogenesis and synaptogenesis.[30] Regulation of these processes was shown to be disrupted by factors affecting the interaction between 5-HT and its receptors and/or transporter (SERT), such as stress and glucocorticoids,[31] and drugs such as cocaine, which inhibit the activity of SERT.[23,32] Upon its direct action on SERT, cocaine exposure evokes glucorticoid release, known to upregulate SERT expression in the developing rat.[31,33] Cerebellar Purkinje cells were shown to present 5-HT receptors;[34] however, to our knowledge, the presence of the SERT has never been described. However, non-serotonergic neurons were reported to express SERT in a transient way throughout embryonic ages and during the first postnatal month.[35,36] Because the enzyme tryptophan hydroxylase, was not detected in any of the structures expressing SERT in a transient way, it was suggested that some cells could express SERT transitorily to use 5-HT as a "borrowed" neurotransmitter.[35]

Serotonin is known to modulate GABAergic synaptic impulses into the cerebellar Purkinje cells.[37] Also, it was reported that 5-HT facilitates ethanol-induced depression of cerebellar Purkinje cells, mainly in young animals,[38] and that differences in

the sensitivity to ethanol of Purkinje neurons correlate with behavioral sensitivity to ethanol-induced ataxia.[39] In addition, exposure to ibogaine, an alkaloid like cocaine, was shown to cause degeneration of the Purkinje cells, associated with hallucinations, tremor, and ataxia.[40] Likewise, the abnormal presence of 5-HT in the Purkinje cells, reported in the present work, seems to indicate that the standard development of these neurons has been affected by neonatal cocaine exposure, with possible implications in its function. In fact, abnormal expression of tyrosine hydroxylase immunoreactivity in the Purkinje cells was shown to precede the onset of ataxia in dilute-lethal mice.[41] Interestingly, we have also observed that the animal that apparently presented more abundant 5-HT–positive Purkinje cells also presented tyrosine hydroxylase–positive Purkinje cells.

Simultaneously, we have observed increased levels of 5-HT in the cerebellum of PND21 pups neonatally exposed to cocaine. At the same time, we have also found increased levels of 5-HT in other brain regions (raphe nuclei, ventral mesencephalon, striatum, nucleus accumbens, and frontal cortex), together with altered exploratory behavior and global activity (manuscript in preparation). Excessive 5-HT release in the frontal cortex and hippocampus was reported to lead to memory impairment,[42] whereas increased extracellular 5-HT levels in the dorsal raphe nucleus, nucleus accumbens,[43] and prefrontal cortex or hippocampus[44] were seen to induce hyperactivity. In the present work, the peak in 5-HT levels in the cerebellum appears soon after the period of final maturation of the Purkinje cells,[9] coincidentally at the end of the critical period for motor development,[45] reinforcing the notion that the abnormal immunoreactivity of Purkinje cells to 5-HT may be linked to alterations of the motor function.

In summary, we report here an abnormal presence of 5-HT in the Purkinje cerebellar cells after neonatal exposure to cocaine, which occurs concomitantly with increased levels of 5-HT in the cerebellum, and may contribute to motor dysfunction.[18,19] Further study is necessary to clarify whether this abnormal immunoreactivity is caused by transient expression of SERT, by unexpected presence of tryptophan hydroxylase, or by additional mechanisms.

ACKNOWLEDGMENTS

We thank Professor John Parnavelas, University College London, London, U.K., for the anti-5-HT antibody. This work was supported by project PRAXIS/PSAU/P/SAU/8/96, by project POCTI/ESP/43630/1999, and by Programa de Financiamento Plurianual do IBMC. Teresa Summavielle received a Ph.D. grant from Fundação para a Ciência e Tecnologia (PRAXIS/BD/14742/97), and Cecília J. Alves a research grant from Fundação para a Ciência e Tecnologia (POCTI/43630-pr 381101).

REFERENCES

1. FRANK, D. *et al.* 2001. Growth, development, and behavior in early childhood following prenatal cocaine exposure: a systematic review. J. Am. Med. Assoc. **285:** 1613–1625.
2. LEWIS, M. & M. BENDERSKY. 1995. Mothers, Babies and Cocaine: The Role of Toxins in Development: 397. Lawrence Erlbaum. Hillsdate, NJ.

3. MAYES, L.C. 1999. Developing brain and in utero cocaine exposure: effects on neural ontogeny. Dev. Psychopathol. **11:** 685–714.
4. WATERHOUSE, B.D. *et al.* 1991. Cocaine actions in a central noradrenergic circuit: enhancement of cerebellar Purkinje neuron responses to iontophoretically applied GABA. Brain Res. **546:** 297–309.
5. LAFORGE, K.S. *et al.* 2003. "Binge" cocaine differentially alters preproenkephalin mRNA levels in guinea pig brain. Brain Res. Bull. **59:** 353–357.
6. GOTTSCHALK, P.C. & T.R. KOSTEN. 2002. Cerebral perfusion defects in combined cocaine and alcohol dependence. Drug Alcohol Depend. **68:** 95–104.
7. JIMENEZ-RIVERA, C.A. *et al.* 2000. Effects of intravenous cocaine administration on cerebellar Purkinje cell activity. Eur. J. Pharmacol. **407:** 91–100.
8. VOLKOW, N.D. *et al.* 1991. Metabolic studies of drugs of abuse. NIDA Res. Monogr. **105:** 47–53.
9. BAYER, S.A. & J. ALTMAN. 1985. Neurogenesis and neuronal migration. *In* The Rat Nervous System. G. Paxinos, Ed.: 1041–1078. Academic Press. New York.
10. DLUGOS, C.A. & R.J. PENTNEY. 2000. Effects of chronic ethanol consumption on SER of Purkinje neurons in old F344 rats. Alcohol **20:** 125–132.
11. SAKATA-HAGA, H. *et al.* 2001. Abnormalities of cerebellar foliation in rats prenatally exposed to ethanol. Acta Neuropathol. (Berl.) **102:** 36–40.
12. PENTNEY, R.J. & C.A. DLUGOS. 2000. Cerebellar Purkinje neurons with altered terminal dendritic segments are present in all lobules of the cerebellar vermis of ageing, ethanol-treated F344 rats. Alcohol **35:** 35–43.
13. PENTNEY, R.J. & L.J. QUACKENBUSH. 1990. Dendritic hypertrophy in Purkinje neurons of old Fischer 344 rats after long-term ethanol treatment. Alcohol. Clin. Exp. Res. **14:** 878–886.
14. DLUGOS, C.A. & R.J. PENTNEY. 1997. Morphometric evidence that the total number of synapses on Purkinje neurons of old F344 rats is reduced after long-term ethanol treatment and restored to control levels after recovery. Alcohol **32:** 161–172.
15. PAULA-BARBOSA, M.M. & M.A. TAVARES. 1985. Long-term alcohol consumption induces microtubular changes in the adult rat cerebellar cortex. Brain Res. **339:** 195–199.
16. TAVARES, M.A., M.M. PAULA-BARBOSA & E.G. GRAY. 1983. Dendritic spine plasticity and chronic alcoholism in rats. Neurosci. Lett. **42:** 235–238.
17. TAVARES, M.A., M.M. PAULA-BARBOSA & E.G. GRAY. 1983. A morphometric Golgi analysis of the Purkinje cell dendritic tree after long-term alcohol consumption in the adult rat. J. Neurocytol. **12:** 939–948.
18. PAULA-BARBOSA, M.M. *et al.* 1983. Cerebellar cortex ultrastructure in ataxia-telangiectasia. Ann. Neurol. **13:** 297–302.
19. NORTHUP, L.R. 1976. Additive effects of ethanol and Purkinje cell loss in the production of ataxia in mice. Psychopharmacology (Berlin) **48:** 189–192.
20. PALMER, M.R. *et al.* 1987. Genetic correlation of ethanol-induced ataxia and cerebellar Purkinje neuron depression among inbred strains and selected lines of rats. Alcohol. Clin. Exp. Res. **11:** 494–501.
21. OHYAMA, T. *et al.* 2003. What the cerebellum computes. Trends Neurosci. **26:** 222–227.
22. ITO, M. 2002. Historical review of the significance of the cerebellum and the role of Purkinje cells in motor learning. Ann. N.Y. Acad. Sci. **978:** 273–288.
23. MENDLIN, A. *et al.* 1996. Neuronal release of serotonin in the cerebellum of behaving rats: an in vivo microdialysis study. J. Neurochem. **67:** 617–622.
24. CASTON, J. *et al.* 1998. Role of the cerebellum in exploration behavior. Brain Res. **808:** 232–237.
25. SUMMAVIELLE, T. *et al.* 2002. Neonatal exposure to cocaine: altered dopamine levels in the amygdala and behavioral outcomes in the developing rat. Ann. N.Y. Acad. Sci. **965:** 515–521.
26. MAGALHÃES, A., M. TAVARES & L. DE SOUSA. 2002. Postnatal cocaine exposure: effects on behavior of rats in forced swim test. Ann. N.Y. Acad. Sci. **965:** 529–534.
27. ALI, S.F., S.N. DAVID & G.D. NEWPORT. 1993. Age-related susceptibility of MPTP-induced neurotoxicity in mice. Neurotoxicology **14:** 29–34.

28. BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann. Biochem. **72:** 248–254.
29. STEINBUSCH, H.W. 1981. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience **6:** 557–618.
30. LAUDER, J.M. & H. KREBS. 1978. Serotonin as a differentiation signal in early neurogenesis. Dev. Neurosci. **1:** 15–30.
31. MCGRATH, K.E., F.J. SEIDLER & T.A. SLOTKIN. 1997. Convergent control of serotonin transporter expression by glucocorticoids and cocaine in fetal and neonatal rat brain. Dev. Brain Res. **104:** 209–213.
32. AKBARI, H.M. *et al.* 1992. Prenatal cocaine exposure disrupts the development of the serotonergic system. Brain Res. **572:** 57–63.
33. SLOTKIN, T.A. *et al.* 1996. Programming of brainstem serotonin transporter development by prenatal glucocorticoids. Dev. Brain Res. **93:** 155–161.
34. GEURTS, F.J., E. DE SCHUTTER & J.P. TIMMERMANS. 2002. Localization of 5-HT2A, 5-HT3, 5-HT5A and 5-HT7 receptor-like immunoreactivity in the rat cerebellum. J. Chem. Neuroanat. **24:** 65–74.
35. HANSSON, S.R., E. MEZEY & B.J. HOFFMAN. 1999. Serotonin transporter messenger RNA expression in neural crest-derived structures and sensory pathways of the developing rat embryo. Neuroscience **89:** 243–265.
36. ZHOU, F.C., Y. SARI & J.K. ZHANG. 2000. Expression of serotonin transporter protein in developing rat brain. Dev. Brain Res. **119:** 33–45.
37. DEAN, I., S.J. ROBERTSON & F.A. EDWARDS. 2003. Serotonin drives a novel GABAergic synaptic current recorded in rat cerebellar Purkinje cells: a Lugaro cell to Purkinje cell synapse. J. Neurosci. **23:** 4457–4469.
38. JENG, C.H. *et al.* 2000. Serotonergic modulation of ethanol-induced electrophysiological depression in young and aged rats. Alcohol. Clin. Exp. Res. **24:** 1730–1741.
39. SORENSEN, S. *et al.* 1980. Electrophysiological correlates of ethanol-induced sedation in differentially sensitive lines of mice. Science **210:** 1143–1145.
40. O'HEARN, E. & M.E. MOLLIVER. 1993. Degeneration of Purkinje cells in parasagittal zones of the cerebellar vermis after treatment with ibogaine or harmaline. Neuroscience **55:** 303–310.
41. SAWADA, K. & Y. FUKUI. 2001. Expression of tyrosine hydroxylase in cerebellar Purkinje cells of ataxic mutant mice: its relation to the onset and/or development of ataxia. J. Med. Invest. **48:** 5–10.
42. SANTUCCI, A.C., P.J. KNOTT & V. HAROUTUNIAN. 1996. Excessive serotonin release, not depletion, leads to memory impairments in rats. Eur. J. Pharmacol. **295:** 7–17.
43. BUBAR, M.J. *et al.* 2003. Selective serotonin reuptake inhibitors enhance cocaine-induced locomotor activity and dopamine release in the nucleus accumbens. Neuropharmacology **44:** 342–353.
44. TAKAHASHI, H. *et al.* 2000. Serotonergic neurons projecting to hippocampus activate locomotion. Brain Res. **869:** 194–202.
45. WALTON, K.D. *et al.* 1992. Identification of a critical period for motor development in neonatal rats. Neuroscience **51:** 763–767.