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Neuroscience Vol. 35, No. 2, pp. 355–363, 1990
Printed in Great Britain

0306-4522/90 $3.00 + 0.00
Pergamon Press plc

BEHAVIOURAL DEFICITS AND SEROTONIN DEPLETION IN ADULT RATS AFTER TRANSIENT INFANT NASAL VIRAL INFECTION

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Abstract—Dysfunction of subcortical serotoninergic neurons has been implicated in some behaviour disturbances. The serotoninergic neurons in the dorsal and median raphe project widely in the brain. They innervate the olfactory bulbs and can be targets for exogenous agents attacking the olfactory epithelium and bulbs. We report here an injury to the serotoninergic neurons after intranasal infection in 12-day-old rats with a temperature-sensitive mutant of vesicular stomatitis virus. The brain infection was focal and transient. Viral antigens could no longer be detected 13–15 days after infection. In spite of this the animals, as adults, had a severe serotonin depletion in the cerebral cortex and hippocampus, and showed abnormal locomotor and explorative behaviour as well as learning deficits. The neocortex was histologically intact and parameters related to other neurotransmitters such as dopamine, noradrenaline, GABA and acetylcholine showed no marked changes.

A relatively selective damage to serotoninergic nuclei as a result of virus neuroinvasion through a natural portal of entry, may constitute a new pathogenetic mechanism for cortical dysfunction and behavioural deficits.

In the brain certain subcortical nuclei have widespread connections and may serve to modulate the function of the cerebral cortex or basal ganglia.[5] These include serotoninergic neurons in the raphe nuclei, noradrenergic neurons in the locus coeruleus, dopaminergic neurons in the substantia nigra and ventral tegmental area, and cholinergic neurons in the basal nucleus of Meynert, the diagonal bands and the septal nuclei. In some of these nuclei, i.e. the raphe nuclei, the diagonal band and the locus coeruleus, nerve cells project directly to the olfactory bulbs, and can accumulate exogenous substances from these regions by retrograde axonal transport. These nuclei may therefore be especially susceptible to pathogenetic agents that reach the neuroepithelium in the nasal cavity.[34,35] Several neurovirulent viruses may attack the olfactory epithelium and spread anterogradely along olfactory pathways to the brain.[14] We have previously shown that by retrograde transport a virus infection in the olfactory bulbs may cause an early attack on the diagonal bands, the raphe and the locus coeruleus.[20,21] By choosing a slowly replicating temperature-sensitive mutant of vesicular stomatitis virus (VSV) in susceptible infant mice the infection was restricted to these regions and there was no further spread into the neocortex or brainstem as detected by immunohistochemistry. The virus antigen disappeared nine to 11 days post infection (p.i.) and the mice survived without any overt neurological signs of disease. The aim of the present study was to examine the long-term effects of such a transient infection on behavioural parameters, and to correlate such parameters with neurotransmitter contents in the brain.

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Abbreviations: ChAT, choline acetyltransferase; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; GAD, glutamate decarboxylase; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, 5-hydroxytryptamine; HVA, homovanillic acid; NA, noradrenaline; p.i., post infection; VSV, vesicular stomatitis virus.

EXPERIMENTAL PROCEDURES

Subjects

The subjects were female offspring of Sprague–Dawley rats (Alab, Södertälje, Sweden). At the time of behavioural tests they were housed in group cages (three or four animals/cage) under laboratory conditions with a 12 h on 12 h off lighting schedule, with food and water available *ad libitum*.

Viral infection

Five litters of 11–12-day-old rats, weighing 18–24 g (the number of siblings varied from 11 to 15 between the groups) were infected with a temperature-sensitive mutant of VSV designated G41 and described earlier.[32,33] The VSV strain was kindly provided by Dr Mauro Dal Canto, Department of Pathology, Northwestern University, Chicago, Illinois, U.S.A. The mutants were grown in BHK_{21} cells and plaque

titrated as described previously.[20,21] The rats received a drop of the virus suspension into each nostril, i.e. about 3–6 × 10^6 PFU/animal. Other litters remained uninfected and served as controls. The rats were followed regularly for clinical signs of disease and body weight. In one of the infected litters seven of 15 rats died seven to 11 days p.i. and in another three out of 13 rats died nine to 13 days p.i. The other rats survived with no overt signs of neurological illness.

Behavioural tests

One group of animals (infected $n = 8$; control $n = 6$) was tested at 1, 2 and 4 months p.i.; while another group (infected $n = 6$; control $n = 6$) was tested at five and nine months p.i.

Cognitive function of the animals was tested in the Morris water maze task. The Morris water maze provides a behavioural testing procedure to investigate spatial information processing in rats. The maze task requires rats to locate an escape platform hidden just below the water surface in a pool of opaque water.[27] Since no visual or olfactory cues are available within the maze, animals use distal (extra maze) cues to locate the platform. The water maze task has been widely used in investigating the neural basis of spatial learning, and it has been established that lesion of the hippocampus impairs acquisition of the maze task.[27a] The apparatus consisted of a circular pool measuring 140 cm in diameter and 45 cm in height; and painted grey. A platform was placed 1 cm below the surface of the water. The swimming test was performed as described earlier[27] with some modification.[26] The platform was placed at a point in the pool. The animal was gently placed in the pool and the latency to find the platform was recorded. The animals were tested on two consecutive days. Five trials were given daily. If the animal failed to locate the platform within 60 s it was placed on the platform and remained there for 60 s before being given another trial. Two measurements of performance were taken: latency to find the platform and number of failures to reach platform.

To obtain information on locomotor activity and exploratory behaviour, animals were tested in automated activity cages. In addition, the activity test provides information on one of the most elementary forms of learning, namely habituation—the waning of a response as a result of constant stimulation. The apparatus used to register spontaneous motor activity was a plexiglass open field (700 × 700 × 450 mm) equipped with two rows of infralight sensitive photocells.[2] Interruptions of photocell beams were collected by means of a microcomputer and allowed the recording of the following variables: (1) peripheral locomotion—horizontal movement along the walls of the open field box; (2) central locomotion—horizontal movement of the animal at the centre of the open field; (3) peripheral rearing—vertical activity along the walls of the apparatus; (4) central rearing—vertical activity at the centre of the arena.

The animal was gently placed in the open field and its spontaneous motor activity recorded every 15 min for 1 h. Four activity boxes were used simultaneously.

Following behavioural tests one group of animals was killed five months p.i. for neurochemical and histological analysis.

Neurochemistry

The concentrations of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), noradrenaline (NA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) were simultaneously assayed by use of high performance liquid chromatography with electrochemical detection according to a previously described method.[25] The concentrations of glutamate decarboxylase (GAD) and choline acetyltransferase (ChAT) were determined as described earlier.[9,10]

Histology

The rats were perfused via the ascending aorta with 4% paraformaldehyde in phosphate buffer. The brains were dissected, embedded in paraffin, and 6-μm-thick sections were cut at regular intervals through the olfactory bulbs, forebrain and brainstem. From rats taken for biochemical analyses a slice through the brain including the rostral part of the hippocampus, and the remainder of the basal forebrain and the brainstem were fixed by immersion and embedded in paraffin. The sections were stained with haematoxylin–eosin or reacted for acetylcholinesterase.

Statistical analysis

The data on the Morris maze test were analysed with two-way ANOVA. *Post hoc* comparisons were made with Newman–Keul's test, provided the ANOVA showed significant overall effect. The number of beam interruptions in the activity test were analysed with a two-factor ANOVA with the different groups as the independent factor and time as the repeated measure.[40] When significant interactions were observed, analysis of simple main effects comparing the groups at different time periods was carried out.[40] For neurochemical data differences in monoamine, GAD and ChAT concentrations between the groups were evaluated with Student's t-test.

RESULTS

The infected rats showed a slight (less than 10%) reduction in body weight.

Histology

Histological examination of six brains sampled from surviving rats from the different litters 13–15 days p.i. showed focal necrosis and leucocytic cell infiltration in the olfactory bulbs, the olfactory tubercles, and in the vertical and horizontal diagonal bands. There was no total necrosis of any of these areas. Necrosis was also seen in the midline of the mesencephalon and upper pons corresponding to raphe. In addition, there were signs of neuronal destruction in the locus coeruleus, although most of the neurons appeared to be intact. Immunostaining using a previously described method[20,21] for virus antigens was negative. The rats taken for neurotransmitter examinations five months p.i. showed remnants of necrosis in these areas, but the extent of the necrotic foci varied between individual rats. The cerebral cortex and hippocampus were intact. Acetylcholinesterase staining showed no apparent reduction in density of nerve fibres as compared with controls, and nerve cell bodies in the nucleus basalis were present, but a quantitation of these neurons was not performed. The infected rats showed neuronal necrosis within the olfactory pathway, the diagonal band, raphe nuclei and locus coeruleus, while the cerebral cortex and hippocampus were intact.

Neurochemistry

VSV infection caused a significant reduction of serotonin and 5-HIAA in the frontal, parietal and occipital cortex, the olfactory bulb and the hippocampus, while the levels in the caudate nucleus and the hypothalamus were not significantly reduced (Table 1). The levels of DA, DOPAC and HVA were

Table 1. Effects of vesicular stomatitis virus infection in rats during infancy on levels of monoamines, monoamine metabolites, choline acetyltransferase and glutamate decarboxylase in different brain regions at five months p.i.

	Serotonin	5-HIAA	Noradrenaline	Dopamine	HVA	DOPAC	ChAT	GAD
Frontal cortex								
Control	6.9 ± 0.44	3.7 ± 0.38	2.7 ± 0.15	0.58 ± 0.04	0.12 ± 0.01	0.15 ± 0.02	27.3 ± 0.9	142.3 ± 10.8
VSV-infected	2.8 ± 0.94**	1.7 ± 0.51*	2.0 ± 0.26	0.43 ± 0.06	0.12 ± 0.01	0.12 ± 0.01	23.4 ± 0.8	130.4 ± 6.2
Parietal cortex								
Control	4.4 ± 0.52	2.9 ± 0.26	2.8 ± 0.17	1.9 ± 1.0	0.34 ± 0.06	0.47 ± 0.12	28.6 ± 4.1	173.9 ± 33.8
VSV-infected	1.6 ± 0.46**	1.2 ± 0.35**	2.1 ± 0.26	1.2 ± 0.25	0.26 ± 0.03	0.27 ± 0.04	26.0 ± 3.0	142.3 ± 7.8
Occipital cortex								
Control	3.8 ± 0.33	2.7 ± 0.25	2.2 ± 0.12	0.20 ± 0.02	N.D.	0.07 ± 0.01	23.4 ± 0.7	142.3 ± 6.1
VSV-infected	1.4 ± 0.30***	1.1 ± 0.25**	1.6 ± 0.19*	0.15 ± 0.01	N.D.	0.07 ± 0.01	23.4 ± 1.2	142.3 ± 6.1
Olfactory bulb								
Control	2.8 ± 0.11	1.7 ± 0.13	2.2 ± 0.11	0.53 ± 0.08	0.43 ± 0.06	0.19 ± 0.04	18.2 ± 1.4	177.9 ± 14.2
VSV-infected	1.4 ± 0.29**	0.88 ± 0.21**	1.6 ± 0.27	0.42 ± 0.07	0.33 ± 0.05	0.20 ± 0.03	18.2 ± 1.5	177.9 ± 13.1
Nucleus caudatus								
Control	5.1 ± 0.78	5.7 ± 0.82	0.36 ± 0.03	66 ± 5.8	5.1 ± 0.4	6.9 ± 1.3	116.9 ± 4.8	
VSV-infected	3.3 ± 0.84	3.30 ± 0.91	0.28 ± 0.04	64 ± 7.5	5.0 ± 0.7	4.7 ± 0.70	101.3 ± 3.0	
Hippocampus								
Control	5.5 ± 0.48	5.1 ± 0.43	2.9 ± 0.27	0.29 ± 0.02	N.D.	N.D.	36.4 ± 1.4	162.1 ± 11.0
VSV-infected	2.3 ± 0.59**	2.2 ± 0.51**	2.5 ± 0.29	0.17 ± 0.02**	N.D.	N.D.	33.8 ± 2.1	142.3 ± 8.3
Hypothalamus								
Control	8.2 ± 0.65	6.7 ± 0.48	0.92 ± 0.16	3.6 ± 0.08	N.D.	0.92 ± 0.16		
VSV-infected	9.0 ± 1.2	6.1 ± 0.95	1.1 ± 0.18	4.0 ± 0.23	N.D.	1.1 ± 0.18		

The data are presented as pmol/mg tissue (for monoamines and monoamine metabolites) and μmol/g prot per h (for choline acetyltransferase and glutamate decarboxylase).

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's t-test).

$n = 8$ (VSV-infected) or 6 (control). N.D., not detected.

largely unaffected, except for a reduction of DA in the hippocampus. Similarly, no change in NA levels was seen in the different brain regions, except in the occipital cortex where a significant reduction was seen. There were no significant changes in concentrations of ChAT and GAD in the forebrain of infected rats as compared with controls (Table 1).

Behaviour

During the swim test all rats swam effectively using the normal adult posture. At one month p.i. no difference could be seen between VSV-treated and control animals for escape latency or number of failures to reach platform. Figure 1 shows the mean latency to reach platform at one, two, four and nine months p.i. by VSV-treated and controls. The VSV-infected rats had higher latencies than controls. However, only at four months p.i. did the groups differ significantly [$F(1,12) = 6.6$, $P < 0.05$]. *Post hoc* tests showed that the VSV-infected rats had significantly longer latency to reach the platform than control animals on both the two test days.

The results of motor activity at one month p.i. are shown in Fig. 2. VSV-infected rats showed significantly more central locomotion counts than controls. Pairwise comparison showed that the VSV-infected rats were significantly more active than controls during the last time period. There was a significant group effect for peripheral locomotion counts [$(F(1,12) = 6.5$, $P < 0.05$]. Pairwise comparisons showed that the VSV-infected rats were significantly more active than controls during the last time period. For central rearing counts the group by time interaction effect approached significance [$F(3,36) = 2.8$, $P = 0.06$]. There was a significant group by time interaction effect [$F(3,36) = 8$, $P < 0.001$] for peripheral rearing counts. Analysis of simple main effects showed that compared with controls the VSV-infected rats showed significantly lower rearing scores during the first time period and higher rearing scores during the last time period.

The results of activity at two months p.i. are shown in Fig. 3. A significant group effect was obtained for peripheral locomotion counts [$F(1,12) = 6.9$, $P < 0.05$]. Pairwise comparisons showed that the VSV-infected rats were significantly more active than controls during all the four time periods. There was a significant group by time interaction effect for central rearing counts [$F(3,30) = 3.6$, $P < 0.05$]. This significant interaction effect indicated that the VSV-infected rats, in comparison with controls, had less rearing counts during the first time period and tended to show more rearing counts during the last time period. Furthermore, a significant group by time interaction effect was noted for peripheral rearing counts [$F(3,36) = 3.0$, $P < 0.05$]. Analysis of simple main effects indicated significantly higher rearing scores by VSV-infected rats, as compared with controls, during the second and fourth time periods.

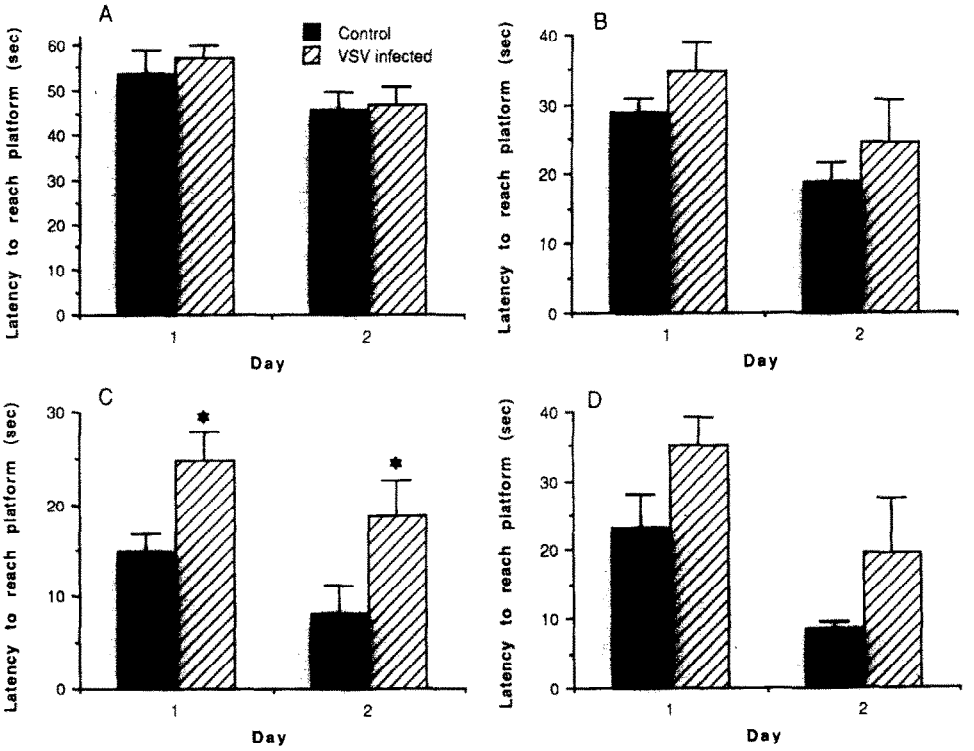

Fig. 1. Acquisition of place learning in the Morris water maze task by VSV-infected ($n = 6$ or 8) and control rats ($n = 6$) at one month (A), two months (B), four months (C) and nine months (D) p.i. Results are presented as mean ± S.E.M. latency to escape onto a hidden underwater platform. *$P < 0.05$.

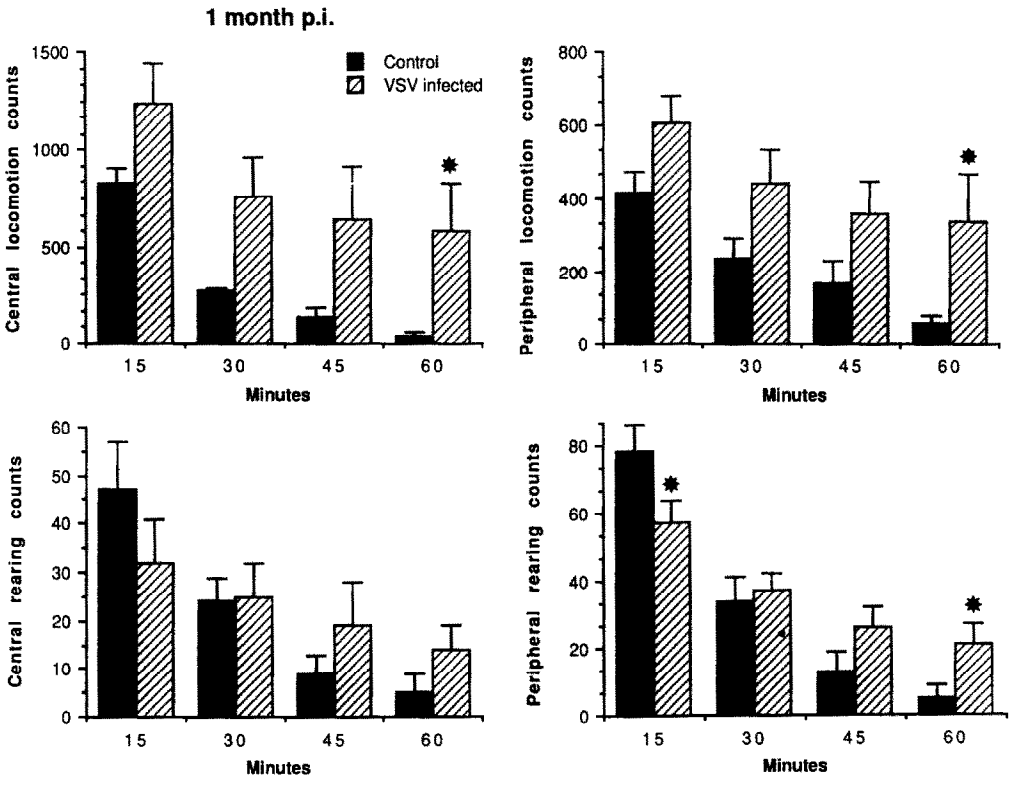

Fig. 2. Effect of VSV infection during infancy on spontaneous motor activity at one month p.i. Results are presented as mean ± S.E.M. locomotion and rearing counts. $^{*}P < 0.05$. $n = 8$ (VSV-infected) or $n = 6$ (control).

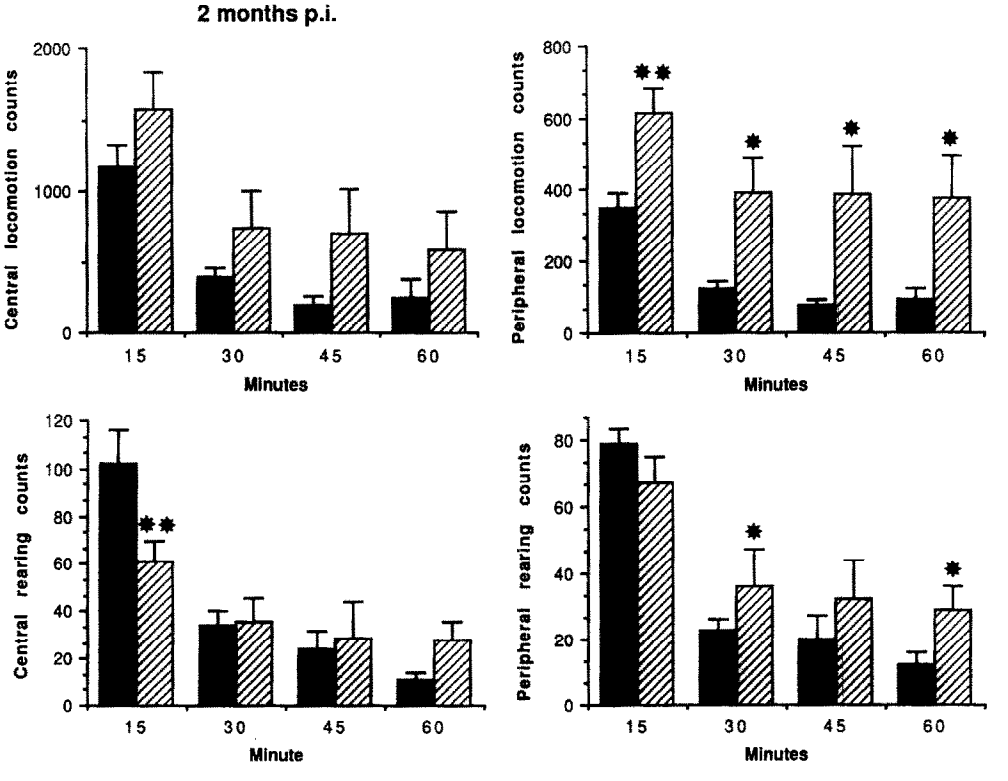

Fig. 3. Effect of VSV infection during infancy on spontaneous motor activity at two months p.i. Results are presented as mean ± S.E.M. locomotion and rearing counts. Solid bars: control; hatched bars: VSV-infected. $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 8$ (VSV-infected) or $n = 6$ (control).

The results from tests on rats at four months p.i. are shown in Fig. 4. There was a significant group difference for peripheral locomotion counts [$F(1,10) = 4.5$, $P < 0.05$]. Pairwise comparisons showed that the VSV-infected rats were significantly more active than controls during the first time period and the last two periods. There was a significant group by time interaction effect for central rearing counts [$F(3,30) = 3.6$, $P < 0.05$]. This significant interaction effect reflected the lower rearing scores of VSV-infected rats during the first time period and higher rearing scores during the last time period. A significant group by time interaction was observed for peripheral rearing scores [$F(3,30) = 3.2$, $P < 0.05$]. Analysis of simple main effects showed that the VSV-infected rats had significantly more peripheral rearing counts than controls during the third time period.

The results from rats at five months p.i. are shown in Fig. 5. For central locomotion counts the group difference approached significance ($P = 0.06$). Furthermore, there was a significant group by time interaction effect [$F(3,30) = 3.9$, $P = 0.01$]. Analysis of the simple main effects showed that the VSV-infected rats were significantly more active than the controls during the last three time intervals. There was a significant group by time interaction effect for peripheral locomotion [$F(3,30) = 3.1$, $P < 0.05$]. Analysis of simple main effect showed that the VSV-infected rats were significantly more active than controls during the second time period. There was a significant group by time interaction for peripheral rearing [$F(3,30) = 3.1$, $P < 0.05$]. It was determined that this interaction effect was due to significantly higher peripheral rearing counts by the VSV-infected rats as compared with controls during the second time period.

The results from tests on motor activity of rats at nine months p.i. are shown in Fig. 6. No significant group differences were obtained.

DISCUSSION

This study has shown that exposure of rats to VSV during infancy can have long-term behavioural and neurochemical consequences. VSV infection during infancy impaired acquisition of the Morris maze task and caused alterations in exploratory behaviour at adulthood. The results indicate that VSV infection causes deficits in associative learning and non-associative learning (habituation).

The behavioural deficits in the VSV-infected rats may be related to the marked serotonin depletion observed in the forebrain, since there is evidence suggesting that serotonin is critically involved in modulation of cognitive functions[1,28,29] and motor activity.[16,39] For example, a selective lesion of serotonin terminals in the prefrontal cortex and the hippocampus by the neurotoxin 5,7-dihydroxytryptamine has been found to impair associative

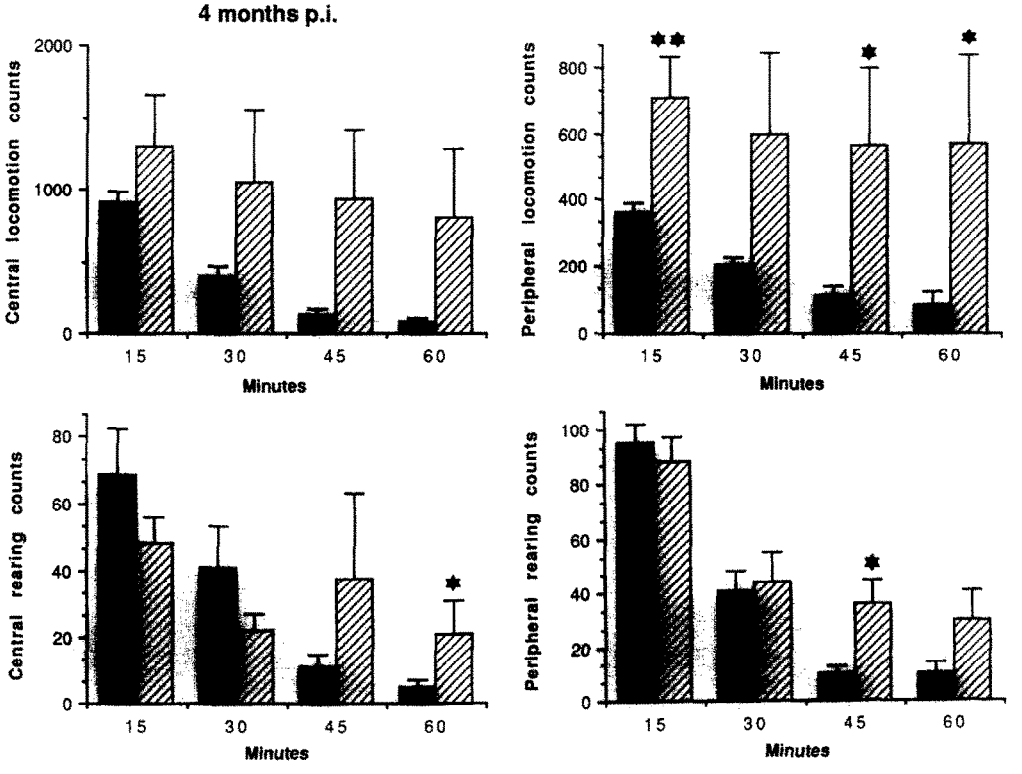

Fig. 4. Effect of VSV infection during infancy on spontaneous motor activity at four months p.i. Details as for Fig. 3. *$P < 0.05$; **$P < 0.01$.

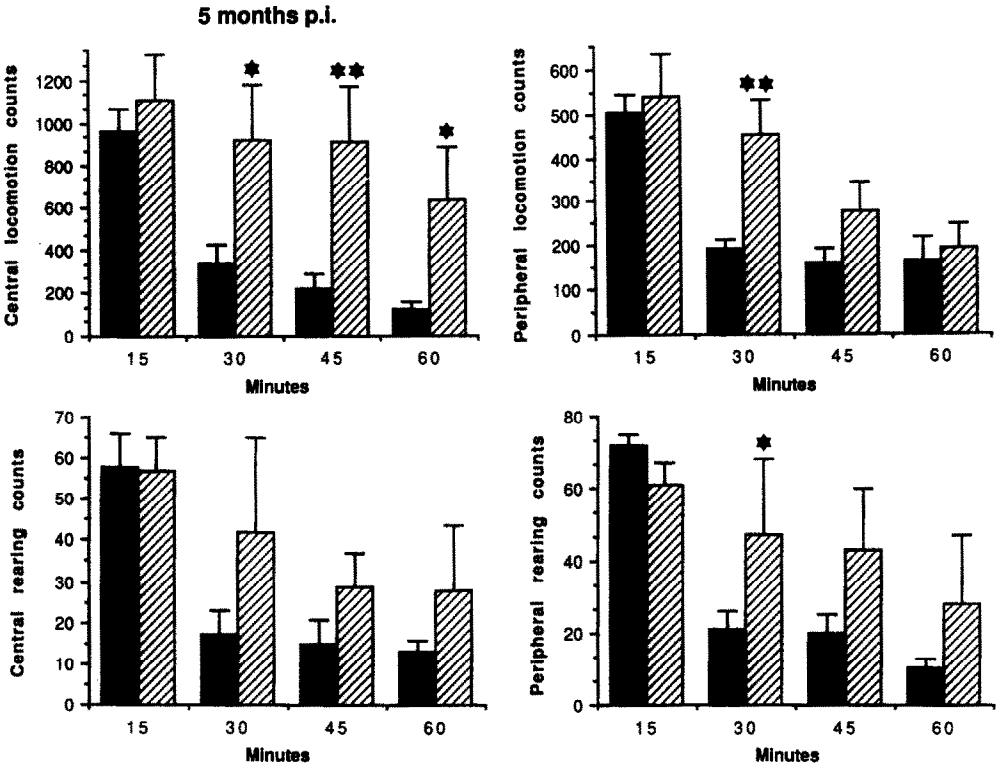

Fig. 5. Effect of VSV infection during infancy on spontaneous motor activity at five months p.i. ($n = 6$ in each group). Details as for Fig. 3. $^{*}P < 0.05$; $^{**}P < 0.01$.

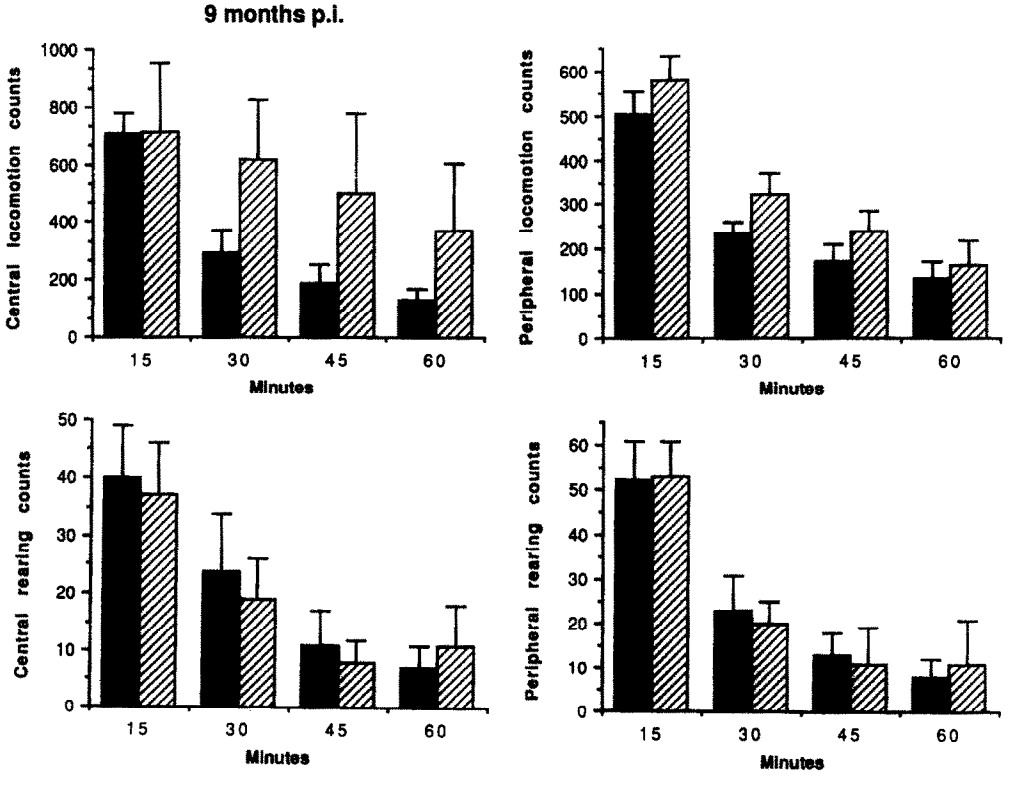

Fig. 6. Effect of VSV infection on locomotion and rearing at nine months p.i. ($n = 6$ in each group). Details as for Fig. 3.

learning,[29] and intraventricular infusion of serotonin can attenuate amphetamine-induced hyperactivity.[39] Neurochemical and behaviour studies suggest the existence of at least two arousal inhibitory systems in the brain, namely a cholinergic and a serotoninergic system.[4,7,24] Removal or pharmacological blockade of one of these systems from the frontal cortex and/or the hippocampus can result in functional impairments of these structures.[3,4,7,12,15,18,19] The VSV-infected animals had a severe loss of cortical and hippocampal serotonin which, thus, may account for the observed hyperactivity. Serotoninergic projections are present in the brain by the 15th day of gestation and the axonal pathways are fully developed at birth.[30] Behavioural studies have shown that in the rat the serotoninergic motor-inhibitory system attains functional maturity at the end of second postnatal week.[4] This correlates with neurochemical findings of increased serotonin synthesis during the same postnatal period.[11,31] The postnatal VSV infection must therefore have destroyed already functionally active aminergic neurons. The serotoninergic midline raphe neurons seem to be more vulnerable to the "hit and run" effect of the VSV than the cholinergic and noradrenergic neurons, although all three systems can be infected.[21] The reason for this is not clear, but may be related to a much higher density of 5-HT terminals in the olfactory bulbs than those of the other neuronal systems analysed,[6] rendering them more likely to be attacked by an exogenous agent in this region.

There have been few behavioural studies of experimental virus infections of the brain. Virtually all of these studies deal with the effects of intracerebral (i.c.) virus inoculations.[13,17,22] For example, i.c. administration of lymphocytic choriomeningitis virus causes a persistent infection in mice and results in long-lasting changes of behaviour characterized by hypoactivity in open field and running wheel activity tests.[17] More selective infections have been observed with a certain strain of mouse hepatitis virus with attack on the substantia nigra and the subthalamic nucleus causing locomotor disturbances.[8] In humans, encephalitis lethargica (supposed to be of viral origin) was associated with alterations not only in the substantia nigra but also in raphe nuclei, locus coeruleus and nucleus basalis of Meynert.[37] In some of the survivors this resulted in Parkinsonism, but hyperactivity and asocial behaviour were also described, especially in children.[38]

CONCLUSION

Our observations show that a virus infection spreading from the nasal cavity at a certain period during infancy can lead to serotonin depletion in the brain and long-lasting alterations of behaviour. The VSV-infected rat may serve as a useful model to evaluate pharmacological treatment strategies for those types of behavioural disturbances, which may have a human counterpart in some cases of attention deficit disorders.[41] Late morphological and biochemical changes due to different types of infections in the olfactory system are also of interest to evaluate, since there are indications that this system is involved early in Alzheimer's disease.[36] Further studies of the effects of self-limiting experimental virus infections established by a natural route of spreading to the brain are warranted. A documentation of corresponding disturbances of cognitive and locomotor functions in other virus–host systems would justify introduction of the term "behavioural virology".

Acknowledgements—This work was supported by grants from the Swedish Medical Research Council (project No. 04480), the Magnus Bergwall Foundation, Loo and Hans Osterman's Foundation for Medical Research, and Greta and Johan Kocks Foundation. The skillful technical assistance of Charlotte Wickman, Åsa Wildte and Gunilla Jöhnemark is gratefully acknowledged. We also thank Inga-Lisa Wallgren for her expert secretarial help.

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(*Accepted* 10 *July* 1989)