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BRAIN RESEARCH

Research Report

Maternal isolation alters the expression of neural proteins during development: 'Stroking' stimulation reverses these effects

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ABSTRACT

Rat pups reared apart from their siblings, mother, and nest environment in the 'pup-in-a-cup' regime show many alterations in behavior reminiscent of the Institutional Inattention/Overactivity Syndrome that characterizes children whose first few months are spent in institutions. In this report, we compare mother-reared (MR) and artificially reared (AR) male rats in concentrations and distributions of brain proteins that are involved in normal brain development. When assessed during the juvenile period and in adulthood, AR animals showed elevations in Neu-N (a neuronal marker) and in S-100 (an astrocyte marker) but reductions in synaptophysin (synapse protein), N-CAM (cell-adhesion molecule), GAP-43 (axon elongation protein), and BDNF (brain derived neurotrophic factor) in comparison to MR controls in many brain sites involved in attention, impulsivity, activity, and social behavior. Daily 'licking-like' stimulation provided to AR animals (AR-MAX) throughout early development that reverses many of the behavioral deficits, also reverses many of the isolation effects on brain proteins. Study 2 showed that elevations in the number of neurons in combination with decreases in functionality are associated with a reduction in neuronal pruning and apoptosis during the very early post-partum period in AR animals and their reversal through daily 'licking-like' stimulation.

1. Introduction

Most young mammals require nurturance from their caregivers (usually the mother) to survive and flourish, and in the absence of that nurturance, undergo disruptions in their long term behavioral and brain development (Li and Fleming, 2003; for a review see Numan et al., 2006).

The effects of maternal separation or deprivation on offspring have been studied most extensively in rodents. Daily maternal separations or single prolonged separations lead to

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Abbreviations: AR, artificially reared; MR, mother reared; AR-MIN, artificially reared, minimal stimulation; AR-MAX, artificially reared, maximal stimulation; MR-CONTROL, mother reared, control; MR-SHAM, mother reared, sham; BDNF, brain-derived neurotrophic factor; NCAM, neural cell adhesion molecule; AMY, amygdala; CA, caudate nucleus; MC, motor cortex; mPFC, medial prefrontal cortex; MPOA, medial preoptic area; NA, nucleus accumbens; VMH, ventromedial hypothalamus; JUV, juvenile; PND, postnatal day; ANOVA, analysis of variance; C, mother reared, control; S, mother reared, sham; MIN, artificially reared, minimal stimulation; MAX, artificially reared, maximal stimulation

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changes in many adult attentional, affective, and emotional behaviors, in stress and metabolic physiology (Francis et al., 2002; Kuhn et al., 1990; Lehmann et al., 2002; Plotsky et al., 2005; Pryce et al., 2001; Rhees et al., 2001; Schmidt et al., 2002), and in patterns of neural development of the autonomic emotional motor circuits (Card et al., 2005) and other brain systems. Some of these maternal separation effects are the result of the absence of the mother rat (Levine, 2002; Macri et al., 2004) and/or the amount of licking provided by her (Lovic and Fleming, 2004; Pauk et al., 1986). Licking during the first few postnatal days maintains cleanliness of and excretion in pups (Gubernick and Alberts, 1983). It also has multiple effects on general growth metabolism (Levine, 1994; Schanberg et al., 1984), development of the HPA axis and neurochemistry (Caldji et al., 1998;

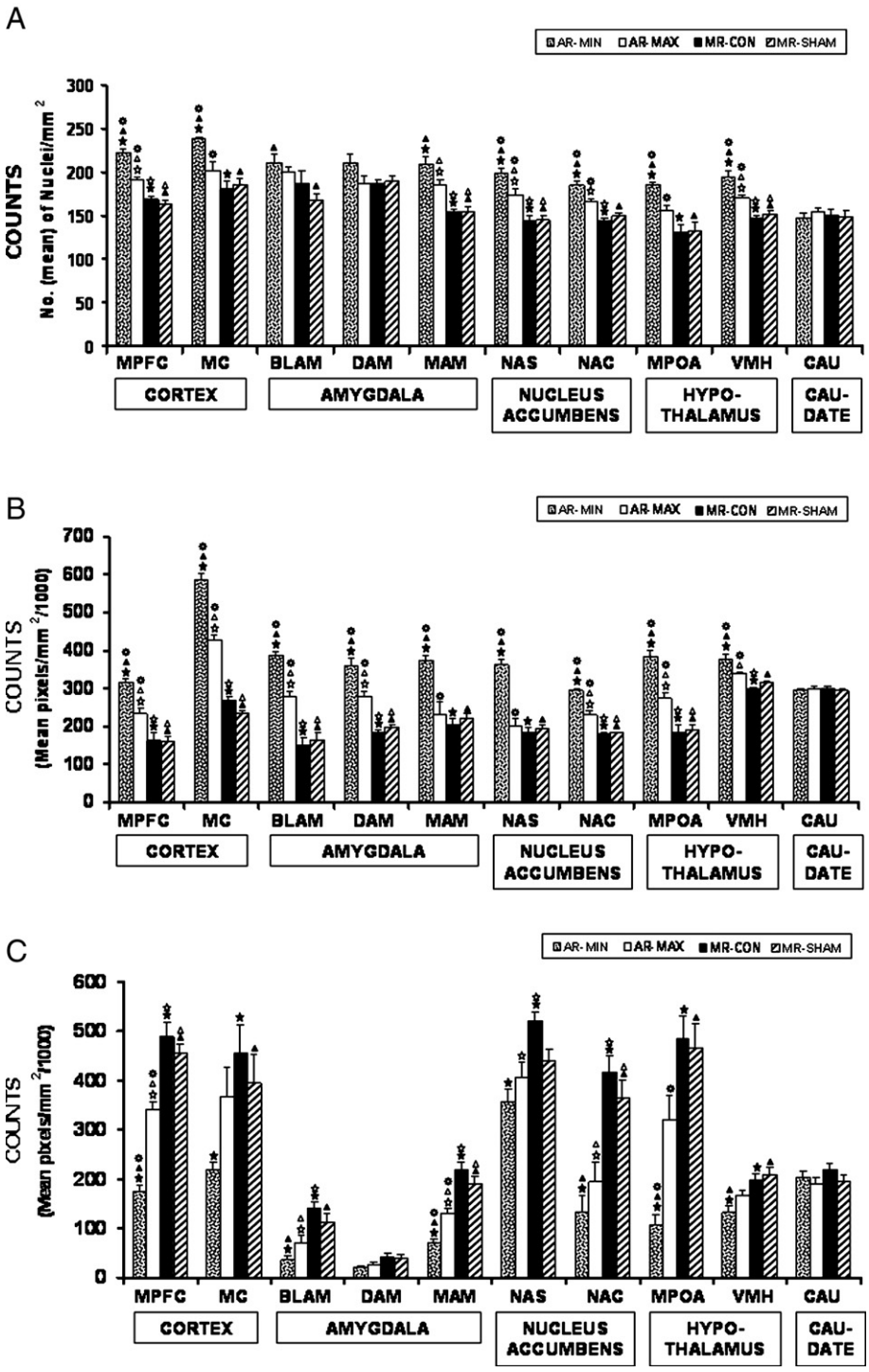

Fig. 1 – Statistical analysis of (A) the number of neuronal cells (Neu-N immunostaining), (B) astrocyte density (S-100 immunostaining), (C) synaptophysin immunostaining, (D) NCAM immunostaining, and (E) GAP-43 immunostaining in different areas of MR-CONTROL (MR-CON), MR-SHAM, AR-MIN, and AR-MAX juvenile rat brains. Results are the mean + SE of seven independent sets. ★ MR-CON vs. AR-MIN, ☆ MR-CON vs. AR-MAX, ▲ MR-SHAM vs. AR-MIN, △ MR-SHAM vs. AR-MAX, ✡ AR-MIN vs. AR-MAX.

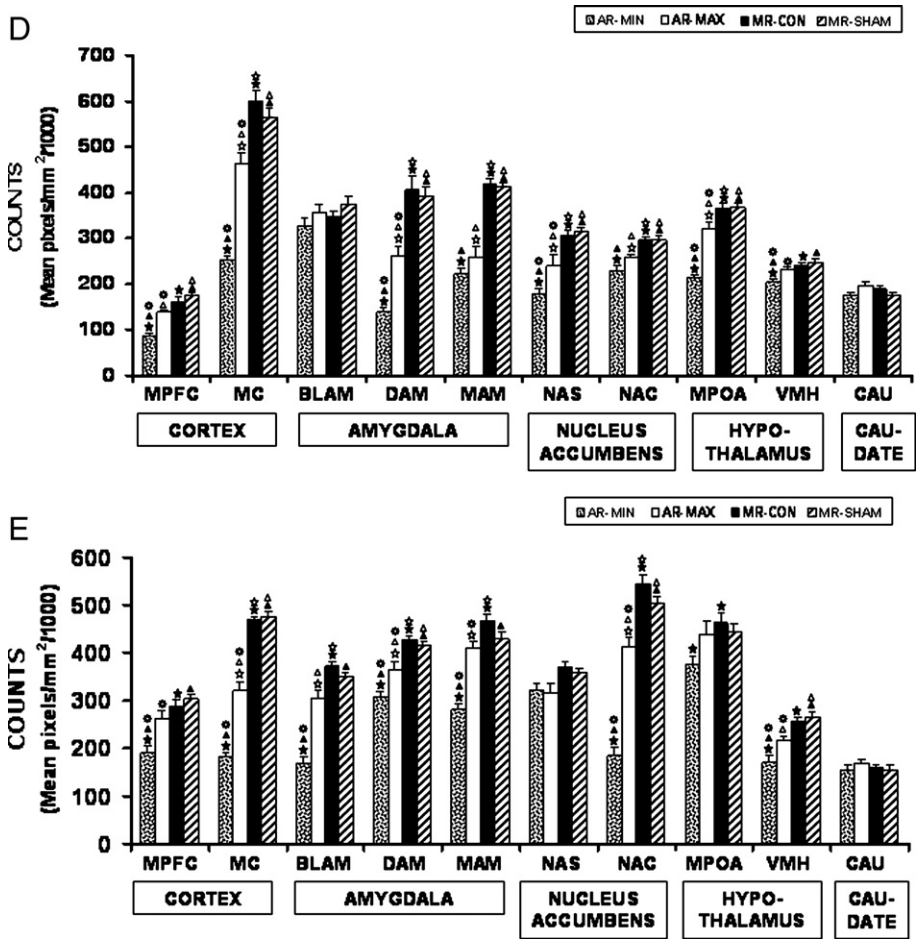

Fig. 1 (*continued*).

Champagne et al., 2003; Francis and Meaney, 1999; Levine et al., 1991, Liu et al., 1997; Weaver et al., 2004b), and development of sexually dimorphic physiology (Moore, 1982, 1984) and behavior (Liu et al., 2000).

When pups are raised entirely without their mothers and littermates, as adults they become hyperactive and show reduced 'fear' in a plus maze task (Burton et al., 2006), exhibit marked inattention in prepulse inhibition and attention set-shifting tasks (Lovic and Fleming, 2004), show enhanced 'impulsivity' (Lovic and Fleming, unpublished data), and display deficits in social learning (Lévy et al., 2003; Melo et al., 2006) and social behaviors (Gonzalez et al., 2001; Lovic and Fleming, 2004) in comparison to mother-reared siblings. For many of these behaviors, tactile stimulation (provided by stroking the pup with a paintbrush 5–8 times per day) can partially or completely reverse these effects (Fleming et al., 2002; Gonzalez et al., 2001; Lévy et al., 2003). Interestingly, these behavioral effects of deprivation are similar in many important respects to the Institutional Inattention/Overactivity syndrome seen in infants raised in institutions who are then adopted into enriched homes (Fries and Pollak, 2004; Gunnar et al., 2001; Rutter and O'Connor, 2004).

Given these extensive maternal deprivation effects on behavior and their reversal with 'stroking', the present studies were designed to determine whether changes occur in molecular markers and indices of brain plasticity in artificially reared (AR) and maternally reared (MR) rats. A second purpose was to determine whether 'licking-like' stimulation reverses these effects. The extensive changes found in behavior due to artificial rearing procedures suggest that structural brain changes should also occur (Akbari et al., unpublished data; Gonzalez and Fleming, 2002; Monfils et al., 2005) in systems mediating effected behaviors.

For the present studies, we evaluated the expression of several neural proteins that are important for postnatal rat brain development and cellular functions in the juvenile and adult brain. In study 1, we used two brain structural proteins, Neu-N and S-100, to quantify neuronal cell number and astrocyte cell marker intensity, respectively (Ingvar et al., 1994; Gittins and Harrison, 2004) and protein markers that reflect synapse integrity (synaptophysin, Masliah et al., 1991; Thiel, 1993), cell–cell communication (NCAM, Cremer et al., 1998; Ronn et al., 1998), axonal path finding (GAP-43[1], Benowitz et al., 1990; Irwin

[1] GAP-43 is the protein expressed primarily at an early age, during axonal and dendritic elongation (Benowitz and Routtenberg, 1997) and undergoes a decrease across the brain during early development (Karimi-Abdolrezaee et al., 2002; Harry et al., 2000; Hulsebosch et al., 1998; Hughes-Davis et al., 2005; Schauwecker et al., 1995). Our attempts to assay this protein marker in the targeted areas in adulthood resulted in extremely low levels of expression. As such, we decided to assay GAP-43 only in the juveniles. BDNF clearly plays a role in the maintenance of synaptic functions and plasticity in adult animals (Thoenen, 1995; Jankowsky and Patterson, 1999; Yamada et al., 2002; Marini et al., 2004); therefore, in these studies we carried out assays for BDNF in adulthood. The absence of assays of juvenile brains was due to practical constraints.

and Madsen, 1997), and neurotrophic activity (BDNF[1], Alderson et al., 1990; Thoenen, 1995). Many of these factors have been implicated in long-term behavioral changes (Lüthi et al., 1994; Muller et al., 1996; Tyler et al., 2002).

In study 2, we attempted to determine whether any early experience induced changes in neuron number seen in juvenile and/or adult animals were due to changes in programmed cell death or apoptosis during postnatal development. Since the peak time for apoptosis in normal development occurs during the early postnatal period (Oppenheim, 1991), we assessed apoptosis on postnatal day (PND) 7 in groups of animals who were either raised with (MR) or without (AR) their mother and littermates during the first week of life (Becker and Bonni, 2004; Nunez et al., 2001; Oppenheim, 1991).

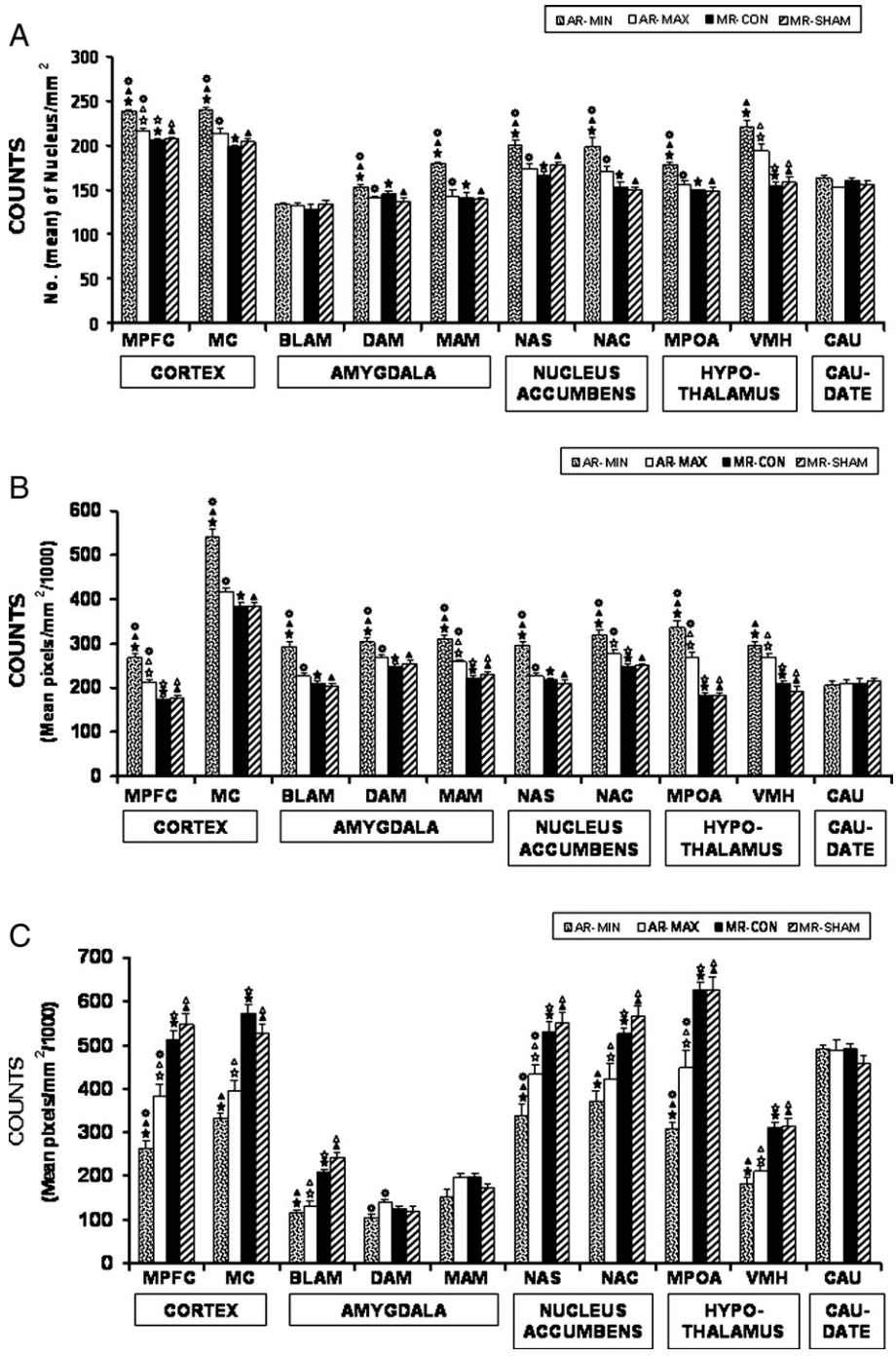

Fig. 2 – Statistical analysis of (A) the number of neuronal cells (Neu-N immunostaining), (B) astrocyte density (S-100 immunostaining), (C) synaptophysin immunostaining, (D) NCAM immunostaining, and (E) BDNF immunostaining in different areas of MR-CONTROL (MR-CON), MR-SHAM, AR-MIN, and AR-MAX adult rat brains. Results are the mean + SE of 7 independent sets. ★ MR-CON vs. AR-MIN, ☆ MR-CON vs. AR-MAX, ▲ MR-SHAM vs. AR-MIN, △ MR-SHAM vs. AR-MAX, ✡ AR-MIN vs. AR-MAX.

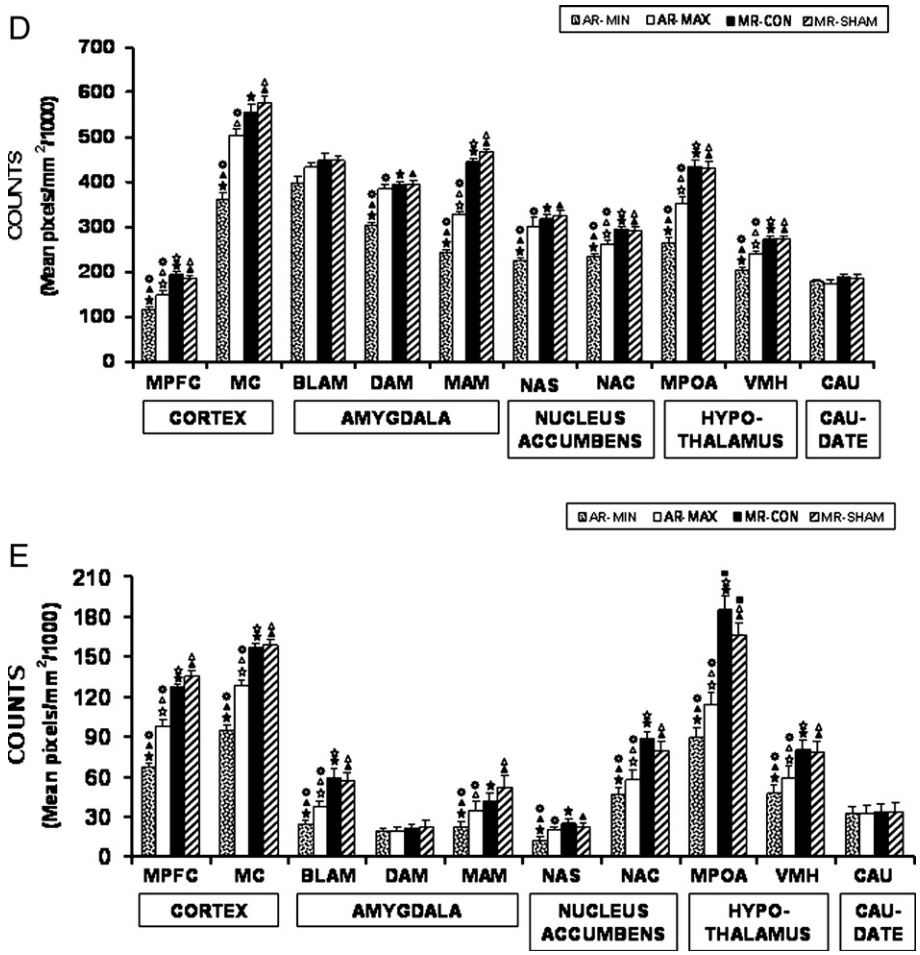

Fig. 2 (*continued*).

The brain areas of interest are those involved in the circuitry underlying maternal, sexual, and other species-typical behaviors found to be affected by early maternal deprivation in our lab, as well as others (Gonzalez et al., 2001; Lovic and Fleming, 2004). These include the motor cortex (MC; movement), medial prefrontal cortex (mPFC; attention and impulsivity), medial preoptic area (MPOA; maternal and sexual behaviors), ventromedial hypothalamus (VMH; sexual behavior), nucleus accumbens (NA; reinforcement and memory consolidation), and amygdala (AMY; stimulus salience, emotional behavior and social learning) (for a review see Numan and Insel, 2003; Numan et al., 2006). The caudate nucleus (CA) served as a control site because the caudate has not been specifically implicated in the behavioral systems known to be affected by early maternal deprivation.

2. Results

Four groups of animals were assessed during the juvenile and adult age periods. At each age the following groups were assessed: (i) AR-MIN (artificially reared with 2 anogenital stimulations per day); (ii) AR-MAX (artificially reared with 2 anogenital stimulations and 8 tactile body stimulations per day); (iii) MR-SHAM (mother reared and underwent surgery to control for possible confounds due to AR surgical procedures); (iv) MR-CONTROL (mother reared and left undisturbed).

2.1. *Study 1: Early experience effects on plasticity proteins in the brain*

2.1.1. *Neuronal cell numbers and astrocyte marker S-100*

Neuronal cell numbers (Neu-N) and astrocyte structural marker intensities (S-100) in different brain areas of the four experimental groups are shown in Figs. 1A and B for juvenile rats and Figs. 2A and B for adult rats. In both cases and for all brain sites, with the exception of the control site (CA), there were significant main group effects (see Table 1). As shown in Figs. 1A and B, post hoc tests for Neu-N showed that for most sites, AR-MIN showed significantly higher numbers of Neu-N cells than both MR groups. MR groups did not differ from one another for either protein or site. Post hoc tests for S-100 found that AR-MIN significantly differed from AR-MAX and both MR groups for all brain sites, except the CA in juvenile rats and the CA and VMH in adult rats.

In contrast, the AR-MAX group showed levels of Neu-N and S-100 that fell between AR-MIN and the two MR groups, differing either from both MR and AR-MIN groups or from only the AR-MIN group (see Figs. 2A and B). Fig. 3A shows the representative photomicrographs of the anti-Neu-N staining in the MPOA region of all four groups in adult rats.

Table 1 – Summary of statistical analyses for all proteins in all brain sites of juvenile and adult rat brains

Brain area		Neonatal	Juveniles					Adults				
	Protein	Apoptosis	GAP43	N-CAM	Neu-N	S-100	Synaptophysin	BDNF	N-CAM	Neu-N	S-100	Synaptophysin
	Site	$df=(3,12)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$	$df=(3,24)$
Cortex	MPFC	**32.048****	**13.299****	**24.601****	**38.203****	**22.253****	**47.938****	**69.241****	**28.783****	**56.438****	**43.03****	**31.148****
		$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
	MC	**29.099****	**152.208****	**67.765****	**12.054****	**143.393****	**3.745***	**65.691****	**43.307****	**18.481****	**33.495****	**31.262****
		$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p=0.024$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Amygdala	BLAM	**23.749****	**58.85****	**1.401**	**3.088***	**46.561****	**12.203****	**65.95****	**2.797**	**0.381**	**21.245****	**47.82****
		$p<0.001$	$p<0.001$	$p=0.267$	$p=0.046$	$p<0.001$	$p<0.001$	$p<0.001$	$p=0.062$	$p=0.768$	$p<0.001$	$p<0.001$
	CAM	**0.177**	**21.626****	**36.277****	**2.471**	**42.958****	**2.593**	**1.155**	**29.593****	**3.857***	**11.1****	**2.739**
		$p=0.910$	$p<0.001$	$p<0.001$	$p=0.086$	$p<0.001$	$p=0.076$	$p=0.347$	$p<0.001$	$p=0.022$	$p<0.001$	$p=0.066$
	MAM	**15.628****	**40.63****	**47.313****	**16.379****	**14.425****	**26.762****	**24.731****	**56.799****	**15.31****	**34.423****	**3.302***
		$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p=0.037$
Nucleus accumbens	NAS	**15.449****	**3.187***	**17.302****	**17.407****	**30.301****	**7.313****	**19.26****	**13.005****	**11.163****	**27.908****	**16.212****
		$p<0.001$	$p=0.042$	$p<0.001$	$p<0.001$	$p<0.001$	$p=0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
	NAC	**17.075****	**81.098****	**15.175***	**27.871****	**59.826****	**15.103****	**61.49****	**28.145****	**10.768****	**19.603****	**12.392****
		$p<0.001$	$p<0.001$	$p=0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Hypothalamus	MPOA	**26.432****	**3.139***	**51.913****	**12.247****	**33.728****	**16.578****	**169.48****	**35.093****	**19.761****	**57.922****	**30.746****
		$p<0.001$	$p=0.044$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
	VMH	**11.640****	**18.713***	**12.71***	**31.751****	**21.53****	**8.225***	**30.769****	**24.352****	**20.632****	**40.257****	**25.026****
		$p=0.001$	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$	$p=0.008$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Caudate	CAU	**0.962**	**0.458**	**3.246**	**0.144**	**0.149**	**0.994**	**0.146**	**0.842**	**1.940**	**0.210**	**0.968**
		$p=0.442$	$p=0.719$	$p=0.081$	$p=0.931$	$p=0.928$	$p=0.443$	$p=0.931$	$p=0.484$	$p=0.150$	$p=0.889$	$p=0.438$

F values are in bold; $p<0.05^{*}$, $p<0.001^{**}$.

2.1.2. *Synaptophysin, NCAM, BDNF, and GAP-43*

The intensity of synaptophysin and NCAM in the different brain areas of the four experimental groups is shown in Figs. 1C and D for juvenile rats and in Figs. 2C and D for adult rats. In all cases and for all brain sites, with the exception of the control site (CA), there were significant main group effects (see Table 1). As shown in Figs. 1C and D and Figs. 2C and D, post hoc tests for synaptophysin and NCAM showed that for the majority of sites, AR-MIN showed significantly lower densities than both the MR groups. Also, the two MR groups did not differ from one another for either protein or site. In contrast, the AR-MAX group showed levels of marker that fell between AR-MIN and the two MR groups. Hence, AR-MAX animals differed either from both MR groups, but not AR-MIN, from AR-MIN only, or from all three groups. Figs. 3B and C show the representative pictures of anti-synaptophysin and

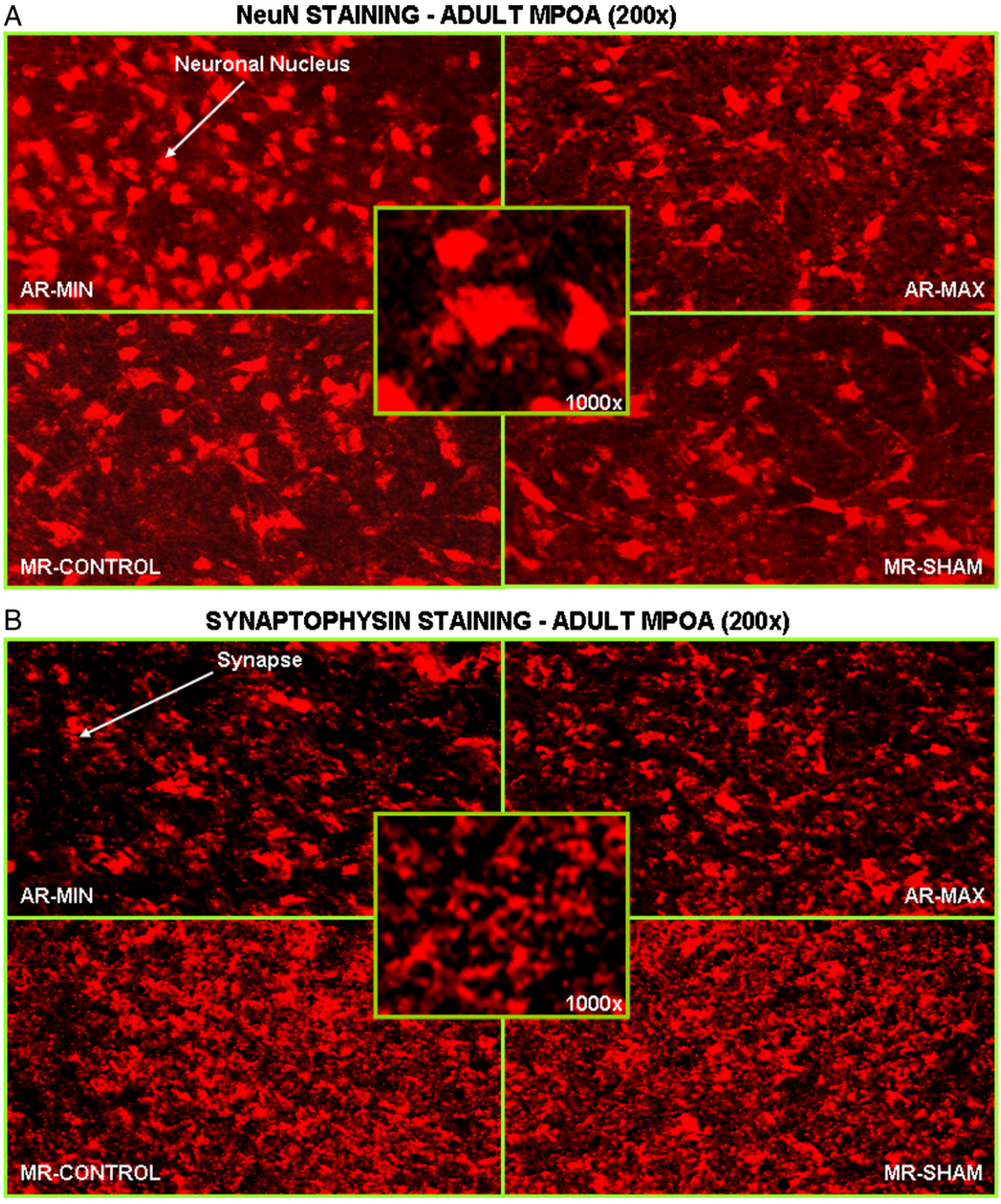

Fig. 3 – Representative photomicrographs showing (A) the number of neuronal cells (Neu-N immunostaining), (B) synaptophysin immunostaining, (C) NCAM immunostaining, and (D) BDNF immunostaining in the MPOA region of MR-CONTROL, MR-SHAM, AR-MIN, and AR-MAX adult rat brains under 20× objective. The immunofluorescence staining pattern of the MPOA region of a MR rat brain with different antibodies at higher magnification (100× objective) is shown as an insert.

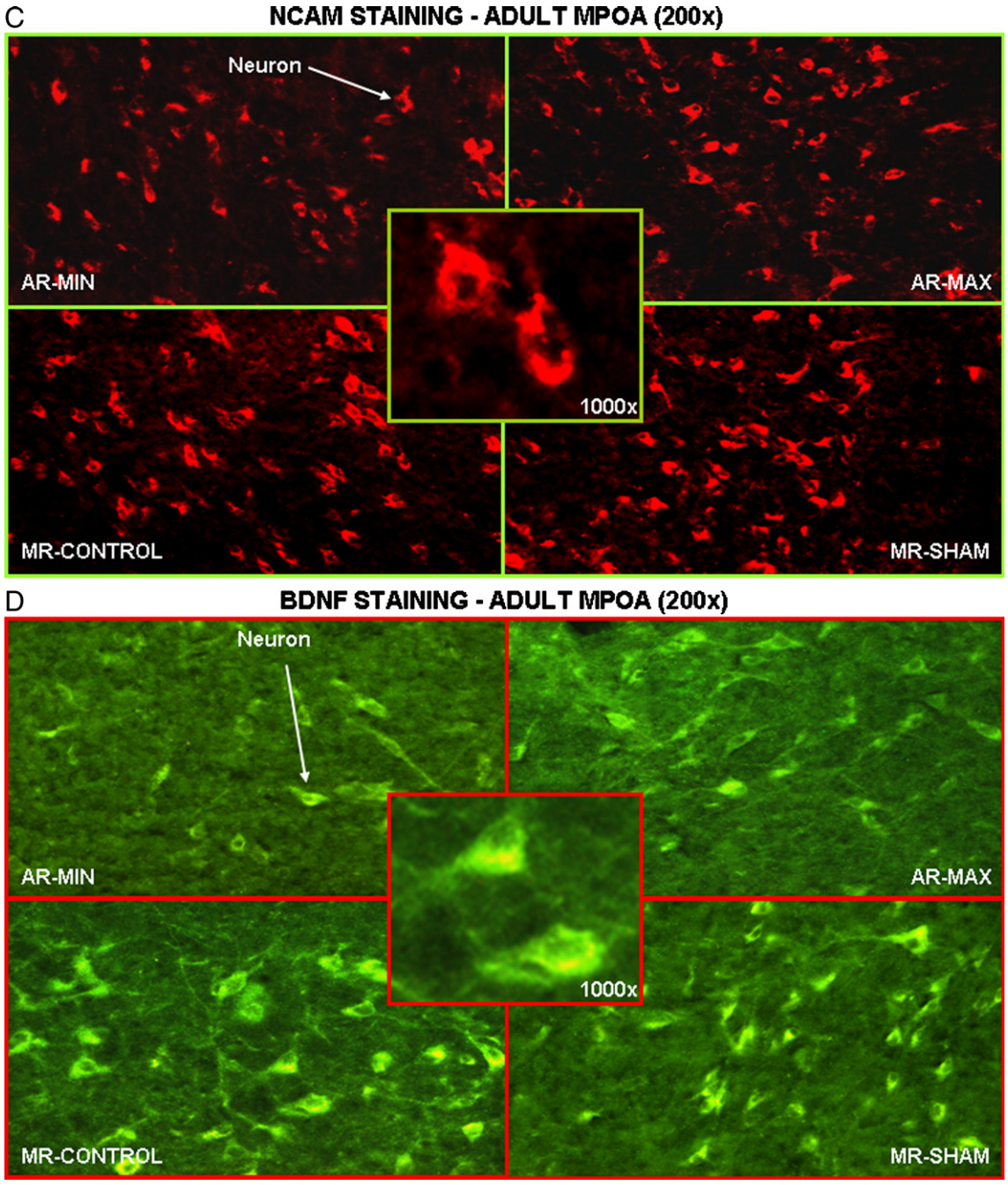

Fig. 3 (*continued*).

anti-NCAM staining in the MPOA region of all four groups of adult animals.

Juvenile brains were also assessed for GAP-43, while adult brains were assessed for BDNF. For both these markers, the pattern was virtually identical as that described for NCAM and synaptophysin (see Figs. 1E and 2E). Hence, there were overall group differences (except in the CA for juveniles and in central AMY and CA for adults) (see Table 1). Post hoc tests indicated that in almost all cases, AR-MIN animals had significantly lower levels than AR-MAX and the two MR groups. The two MR groups did not differ from one another (see Figs. 1E and 2E).

Also, as shown in Figs. 1E and 2E, BDNF expression in AR-MAX groups had levels that were higher than those found in AR-MIN animals but lower than or no different from the MR control groups. AR-MAX animals were significantly different from both MR groups and the AR-MIN group in many regions. The representative pictures of anti-BDNF (in adults) in the MPOA can be seen in Fig. 3D.

2.1.3. *Western blot analysis of synaptophysin, GAP-43, S-100, BDNF, and NCAM proteins*

We further confirmed the results of the above immunohistological analysis by Western Blot analysis using antibodies against synaptophysin, GAP-43, S-100, BDNF, and NCAM in different brain areas of AR and MR juvenile (Fig. 4) and adult (Fig. 5) rats. The Western Blot analysis results were in agreement with the

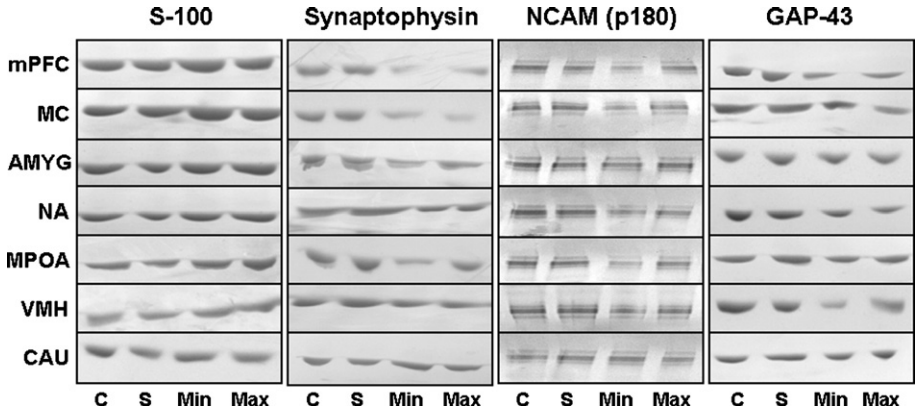

Fig. 4 – Western Blot analysis visualizing the expression of S-100, synaptophysin, NCAM, and GAP-43 in different areas of MR-CONTROL (C), MR-SHAM (S), AR-MIN (MIN), and AR-MAX (MAX) juvenile rat brains.

results of immunohistochemical studies in the different brain regions, both in juvenile and adult brains. In most of the areas studied in the juvenile brains, except in the caudate and VMH, the staining reflecting the expression of synaptophysin, GAP-43, and NCAM was much darker in the two MR groups compared to AR-MIN. However, S-100 staining was darker in AR-MIN animals compared to MR-CONTROL and MR-SHAM animals. Expression of these proteins for AR-MAX animals fell in between the two MR groups and AR-MIN. In adult rats, we also observed similar patterns of expression.

2.2. *Study 2: Early experience effects on apoptosis in the brain*

The intensity of apoptotic cell death or evidence of DNA fragmentation by TUNEL reaction in the different brain areas of the four experimental groups is shown in Fig. 6. In all cases and for all brain sites, with the exception of the control site (CA), there were significant main group effects (see Table 1). As shown in Fig. 6, post hoc tests for TUNEL showed that, for the majority of brain sites, AR-MIN animals showed significantly *lower* densities than both the MR groups. Also, the two MR groups did not differ from one another for any site. In contrast, the AR-MAX group showed levels of apoptotic cell death that fell between AR-MIN and the two MR groups. Hence, AR-MAX animals differed either from both MR groups, but not AR-MIN, from AR-MIN only, or from all three groups. Fig. 7 shows the representative photomicrograph of TUNEL staining in the MPOA region of all four groups of neonatal animals. There was good correspondence between the different groups in apoptotic cell death and neuronal cell numbers in the various brain areas of the juvenile as well as adult AR/MR rat brains (Table 2). For example, in juvenile rats there was 41% less apoptotic cell death and 38% more neuronal cells present in the MPOA of AR-MIN brains compared to MR brains in juveniles. In most of the other brain areas, the percent reduction in apoptotic cell death and percent elevation in neuronal cells in AR-MIN brains compared to MR brains also resulted in good correspondence (viz. NAC 45% vs. 38%; see Table 2). In adults this correspondence existed though there were some recoveries from damage in most of the targeted areas.

3. Discussion

Taken together these results indicate that if rats are deprived of their mothers and usual nest environment during the early postnatal period, brain development is altered such that rats

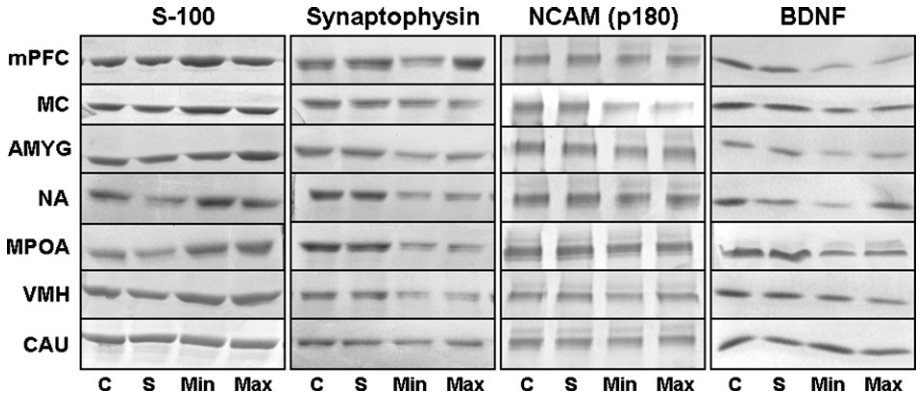

Fig. 5 – Western Blot analysis visualizing the expression of S-100, synaptophysin, NCAM, and BDNF in different areas of MR-CONTROL (C), MR-SHAM (S), AR-MIN (MIN), and AR-MAX (MAX) adult rat brains.

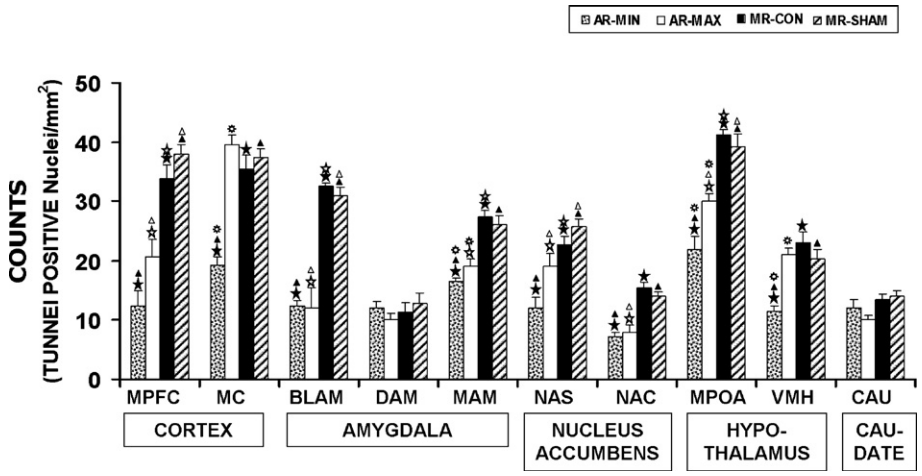

Fig. 6 – Statistical analysis of the number of TUNEL positive cells in different areas of MR-CONTROL (MR-CON), MR-SHAM, AR-MIN, and AR-MAX neonatal (PND 7) rat brains. Results are the mean + SE of 4 independent sets. ★ MR-CON vs. AR-MIN, ☆ MR-CON vs. AR-MAX, ▲ MR-SHAM vs. AR-MIN, △ MR-SHAM vs. AR-MAX, ✡ AR-MIN vs. AR-MAX.

show persistent elevations in the number of neurons and supporting astroglial cells, but reduced indices of functionality in most brain sites investigated. These effects were seen in juvenile rats and persisted into adulthood. Hence, in most brain sites of interest, rats reared without their mothers showed elevated numbers of cells stained for Neu-N (neurons) and S-100 (astrocytes), but reduced staining for synaptophysin and NCAM, proteins that reflect activity of synaptic vesicles and neurite outgrowth, respectively (Cremer et al., 1998; Ronn et al., 1998). Juvenile rats reared without their mothers also showed reduced GAP-43, a protein expressed in early development that is involved in axonal path finding and synaptic plasticity (Benowitz et al., 1990; Irwin and Madsen, 1997). Artificially reared adult rats showed reduced BDNF, a neurotrophic factor that is involved in regulating survival, differentiation, and maintenance functions of specific populations of neurons and other processes of neuronal plasticity (Alderson et al., 1990; Thoenen, 1995). Taken together these effects are consistent with the results of Card et al. (2005) and Rinaman et al. (2000) who found an alteration in the neural development of limbic, cortical, and hypothalamic inputs to the autonomic system in pups that had been separated from their mothers for 15 min or 3 h daily during the first 10 days of life. In this case, disruptions in neural connectivities or assemblies were established through the use of *trans*-synaptic pseudorabies virus (PRV) injection and immunolabeling within the

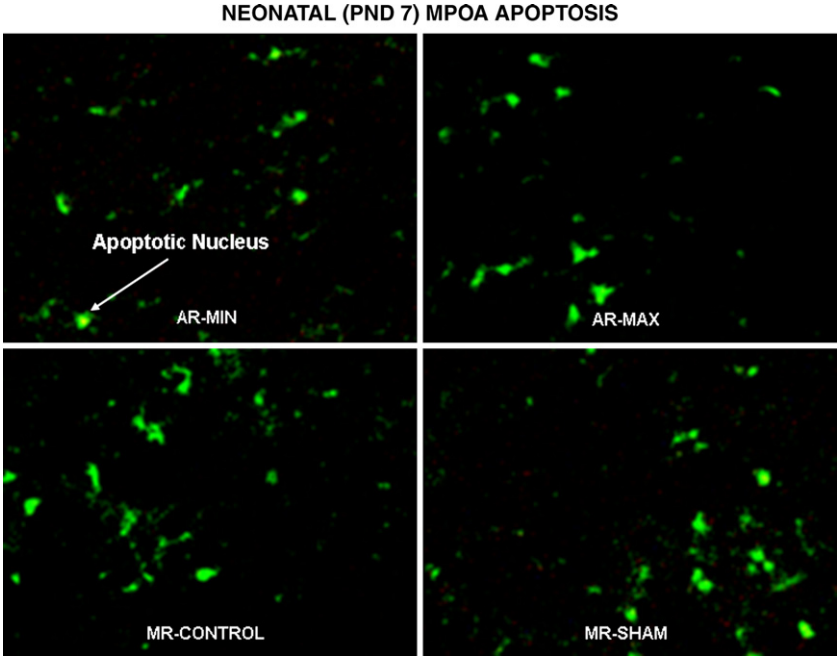

Fig. 7 – Representative photomicrographs representing TUNEL positive cells showing apoptotic nuclei in the MPOA region of MR-CONTROL, MR-SHAM, AR-MIN, and AR-MAX neonatal (PND 7) rat brains under 20× objective.

Table 2 – Results showing correspondence between inhibition of neural apoptosis at PND 7 and increased accumulation of neuronal nuclei in juvenile and adult AR rats compared to MR rats

AGE	MPFC	MC	BLAM	CAM	MAM	NAS	NAC	MPOA	VMH	CAU
Apoptosis										
PND 7	59%	41%	58%	4%	64%	45%	43%	41%	48%	1%
NeuN										
PND 23 juveniles	34%	32%	12%	6%	38%	38%	32%	38%	18%	1%
PND 85 adults	19%	23%	5%	5%	14%	22%	29%	24%	36%	0%

Correspondence between apoptosis and neuronal number:
Percent decrease in apoptosis (TUNEL reaction) between AR-MIN and MR-SHAM on PND 7 in rat brains.
Percent increase in neuronal number (NeuN) between AR-MIN and MR-SHAM in juvenile (PND 23) and adult (PND 85) in rat brains.

circuit. Not all cell groups were equally affected by the early separation regime; primarily those involved in the autonomic-emotional motor circuit.

In the present study, most of the effects of artificial rearing can be reversed, in part or completely, by providing young pups with additional 'licking-like' stimulation during development. These AR-MAX effects are consistent with, and may in part explain, the very elegant findings by Meaney and his colleagues who have described in detail the multiple effects of variations in maternal licking stimulation received by pups during the early postnatal period. Among the effects reported by Meaney and his group are licking-induced differences in various behaviors (such as spatial learning (Liu et al., 2000), fear (Caldji et al., 1998), attention (Zhang et al., 2005), and maternal behavior (Francis et al., 1999)) and in related underlying neuroendocrinology and neurochemistry (Champagne et al., 2003; Zhang et al., 2005). For example, pups receiving a low level of licking show hyperactivity of the HPA stress response and of underlying molecular and epigenetic mechanisms (Liu et al., 1997; Weaver et al., 2004a). Evidence that these effects are based on postnatal experiences and on maternal behavior rather than on prenatal experiences or genetics is indicated by the findings that the effects of low licking stimulation can be reversed through cross-fostering (high to low and low to high licking mothers) (Francis and Meaney, 1999). The Meaney low-high licking distinction is quite similar to the maternal deprivation and stroking replacement comparisons reported here and by Levine and colleagues (Levine et al., 1991; Levine, 2002; Van Oers et al., 1998). Specifically, the similarities lie in the effects on behavior and physiology and are likely mediated by many of the same neural changes during development that we find in the present study.

The present AR effects were seen in most brain sites, but not in all. In a number of sites not involved in the regulation of species-specific behaviors, especially the caudate nucleus, effects of artificial rearing were rarely found. This suggests that although the effects are quite widespread, they are by no means universal. The inverse relation between the structural markers of neurons and astrocytes and functionality of synapses and communication markers indicates that the abundance of neurons present in these early isolates is not functional and hence, is likely not 'normal'.

These results also suggest that the deprivation and replacement regimes have effects on a process occurring early in development, resulting in elevations in the number of neurons and glial cell structural marker intensity. To better understand how the different proteins could affect neuronal development, work on cortical development may constitute an instructive model (Anderson, 2001). For instance, during the development of the cerebral cortex, several events take place leading to the formation of a proper neuronal network in the brain. First, blast cell proliferation generates neuroblasts and glioblasts (during the embryonic period through to PND 2–3 (Meyer et al., 1998; Volkova, 1975; Bayer and Altman, 1987; Jacobson et al., 1985). The neuroblasts and glioblasts then differentiate into mature neurons and various glial cells. During this phase, neuronal migration along glial cell fibers is very important for the organization of neuronal networks and glial cells. In the rat brain, this occurs during the first 2 weeks after birth (Parnavelas, 2000; O'Leary and Koester, 1993). NCAM plays a vital role in this migration. During this period, a substantial number of neurons die to make room for proper network formation, i.e. elongation of axons and dendrites. Apoptotic cell death starts late in the embryonic stage, peaks on PND 7 and then falls sharply by PND 10 (Nunez et al., 2001; Ferrer et al., 1994). GAP-43, BDNF, and NCAM all play an important role during this period, whereas synaptophysin and BDNF are involved in the formation of mature synapses.

To understand the mechanism behind the elevated cell density in AR rat brains, in the second study, we examined apoptotic cell death during brain development in all four groups of rats. We chose PND 7 for the apoptosis study as this is the peak period for neuronal apoptosis in male rat brains (Nunez et al., 2001; Ferrer et al., 1994). This neuronal cell death is not random—it takes place in a very specific manner known as programmed cell death, or apoptosis. During development, the nervous system initially generates an excess number of neurons; thus, apoptosis is an integral process in the normal development of the nervous system. Apoptosis takes place during a restricted period of brain development, leading to the elimination of as much as half of the originally produced neurons (Becker and Bonni, 2004; Oppenheim, 1991). We found, as hypothesized, that there were significantly lower numbers of TUNEL positive cells (apoptotic cells) in many areas of AR-MIN rat brains, in comparison to brains in the MR groups and frequently in the AR-MAX group. These results may then explain the higher number of neuronal cell bodies in AR-MIN animals sacrificed either as juveniles or in adulthood. These results are somewhat different from those reported by Zhang et al. (2002), who reported more cell death after maternal separation. Differences in results likely reflect

differences in the methods of maternal separation used in the two studies. In Zhang et al. (2002), maternal separation consisted of a single 24-h period, whereas in this study, pups were continually reared without a mother. Another major difference was that during the single 24-h separation, pups were completely deprived of food and water. In contrast, in the present study, pups were provided with sufficient milk/food during the entire separation procedure. There have been several reports of induction of apoptosis of neuronal cells after food/glucose deprivation in vivo as well as in vitro (Ferrand-Drake et al., 1999; Ioudina et al., 2004; Wieloch, 1985). Similar inconsistencies have been found for BDNF where Greisen et al. (2005) reported higher levels of BDNF expression in the hippocampus of maternally deprived rat brains, whereas in our study, as well as in that of Roceri et al. (2002), decreased BDNF expression was found.

This is the first direct report of widespread deficient neuronal development in the brains of rats reared in isolation. Using markers of neuronal structure and function, differences in the expression of several important proteins involved in neural development, synapse functions, and neural plasticity were observed. We also found that deficits in processes of neuronal development occur very early during the neonatal period when artificial rearing affects the process of programmed cell death or apoptosis. These results may explain the elevations in neurons found in the artificially reared adult animal. The major achievement of our study is that artificial 'licking-like' stimulation received by the AR-MAX group not only can reverse some behavioral and neuroendocrine abnormalities produced by artificial rearing reported earlier (Fleming et al., 2002; Gonzalez et al., 2001; Gonzalez and Fleming, 2002; Lévy et al., 2003; Lovic and Fleming, 2004; Numan et al., 2006), but can also significantly enhance processes of brain development likely underlying many of these behavioral and physiological effects. This reversal of improper neural network formation by artificial 'licking-like' stimulation raises hope for intervention strategies for infants who experience neglect during early development (Fries and Pollak, 2004; Gunnar et al., 2001; Rutter and O'Connor, 2004). At present we are working on how "stroking" can reverse the adverse effects of isolation rearing on brain development. A number of potential mechanisms exist. However, currently our focus is on the secretion of factors related to skin stimulation (growth factors or cytokines) that are known to influence early brain development; these include FGF, IL6, and TNFα.

4. Experimental procedures

4.1. Statistical analyses

For each marker at each brain site and for each age group, one-way ANOVAs comparing the 4 early experience groups were computed. For some of these analyses, brain weights were also added as a covariate. Where overall group differences were found, post hoc Tukey tests were performed comparing the different pairs of groups. Given the large number of comparisons undertaken, p values of 0.01 were taken as reflecting significant differences for ANOVAs. In general, however, p values <0.005 and higher were found as indicated by Table 1 which includes the F and p values for all analyses completed for the different proteins in juvenile and adult animals and for the different brain sites.

4.2. Animals

4.2.1. Subjects

In the first study, 56 male Sprague–Dawley rats were analyzed either as juveniles ($n=28$) or as adults ($n=28$). In the second apoptosis study, 16 rats were sacrificed on postnatal day (PND) 7. Experimental animals were born and raised at the University of Toronto at Mississauga from a stock originally obtained from Charles River Farms (St. Constant, Quebec, Canada). Rearing conditions are described below. In general, the progenitor colony was maintained on a 12:12 h light:dark cycle with lights on at 0800 h in a room maintained at approximately 22 °C, humidity 50–60%. Beginning on PND 21, all mother rats producing experimental offspring were housed two per cage (clear, 20×43×22 cm), with food (Purina Rat Chow, Brentwood, MO) and water available ad libitum until they were mated, as adults, with stud males. Mother rats were housed singly throughout their pregnancies. All procedures involving animals were approved by the University of Toronto Animal Care Committee and completed in accordance with the guidelines of the Canadian Council on Animal Care.

4.3. General procedures

Mother rats of experimental animals gave birth undisturbed. On day 1 of parturition (PND 1), litters were culled to 10 rats (6 males and 4 females). On PND 3, three male pups were removed from the nest and implanted with a cheek cannulae (see Surgery below) while the remaining three male pups stayed with their four female littermates and the mother rat (MR-CONTROL). One of the three males removed from the nest received a sham surgery and was returned to the nest (MR-SHAM), and the remaining two pups were artificially reared (AR) and randomly assigned to one of two AR groups (see Treatments and Groups below).

4.3.1. Surgery and artificial rearing

Prior to surgery, pups were weighed and a topical anesthetic (EMLA, AstraZeneca, Mississauga, ON) was applied to the surface of the cheek. A lubricated (mineral oil) lead wire (stainless steel, 0.25 mm diameter) with silastic and PE 10 tubing was used to pierce the cheek. The lead wire and silastic tubing were removed once the flared end of the tubing was adjusted appropriately. Polysporin (Pfizer, Toronto, ON) was applied topically to the site of penetration. Another lead wire was used to insert a flat and t-washer which was secured in place with superglue. This same procedure was used for MR-SHAM pups except the PE 10 tubing was removed. Black marker was applied to the ears of MR-SHAM pups prior to placing the pups back with the litter for later identification.

After surgery, AR pups were placed individually in plastic cups (11 cm diameter×15 cm deep) containing corn cob bedding (Bed O'Cobs, Maumee, OH). The cups floated in temperature controlled water (36–40 °C). Animals were connected to time-controlled infusion pumps (Harvard Apparatus Syringe, PHD

2000, St-Laurent, QC) by cheek cannulae tubing. The pumps delivered milk (Messer diet; Messer et al., 1969; Smart et al., 1983) for 10 min every hour, 24 h a day. The amount infused was calculated based on mean pup body weight. Beginning on PND 3, pups received a volume of milk equal to 33% of the mean body weight, and this amount increased by 1% daily. Each morning, pups were disconnected from the pumps, their weights were recorded, and all tubing was flushed with double distilled water. New syringes with fresh formula were prepared and the new infusion rates programmed based on the weights for that day.

4.3.2. *Treatment and groups*

Study 1: AR rats were randomly assigned to either AR-MIN or AR-MAX groups. AR-MIN pups were stimulated twice daily (morning and night) for 30 s each with a wet camelhair paintbrush in the anogenital region to promote urination and defecation. AR-MAX pups received two anogenital stimulations in the same manner as the AR-MIN pups, as well as five 2-min body stimulations daily using a dry camelhair paintbrush. Stimulations for both groups occurred from PND 4 to PND 16. Due to the constraints of the AR procedure (pup-in-a-cup), on PND 18, AR pups were removed from the pumps and given warm milk formula mixed with rat chow powder. The concentration of rat chow powder was slowly increased daily. From PND 20 to 22, AR animals were provided with solid pellets in addition to the milk formula mixed with rat chow powder. On PND 22, all AR pups were only fed solid pellets. MR-pups" were removed from their mothers and fed solid food pellets on PND 22. On PND 22, both AR and MR pups were removed and housed in a cage with a littermate and provided with food pellets and water, ad libitum. Rats in the juvenile group were left undisturbed until PND 23, whereas animals in the adult group remained undisturbed until PND 85. PND 23 was selected as the age for the juvenile groups since this is when most regions of the brain that were studied in this report first attain steady levels of synaptogenesis (Jacobson, 1991; O'Callaghan, 1992; Rice and Barone, 2000).

This resulted in the following group compositions: JUV: AR-MIN, $n=7$; JUV: AR-MAX, $n=7$; JUV MR-SHAM, $n=7$; JUV MR-CONTROL, $n=7$; ADULT AR-MIN, $n=7$; ADULT AR-MAX, $n=7$; ADULT MR-SHAM, $n=7$; ADULT MR-CONTROL, $n=7$. Rats were taken from 14 litters. All litters were represented among the groups such that no more than one animal from each litter was used in each group.

Study 2: All groups and conditions were the same as in Study 1, with the exception of the duration of rearing manipulations which occurred from PND 3 to 7. Animals were sacrificed on PND 7. This resulted in the following group compositions: AR-MIN, $n=4$; AR-MAX, $n=4$; MR-SHAM, $n=4$; MR-CONTROL, $n=4$. Four pups were used per group each deriving from a different litter.

4.4. *Procedures prior to assays*

Study 1: Brain samples were taken at two ages: the juvenile period (PND 23) and in adulthood (PND 85). Rats sampled during the juvenile period were sacrificed following full weaning, classified as when the animals were able to eat solid food. Rats sampled during adulthood were paired with a same sex mother-reared conspecific until they were sacrificed. After decapitation, brains were extracted and quickly frozen on dry ice and stored at −80 °C until assayed with immunohistochemical procedures and Western Blot analyses.

Study 2: Brain samples were taken during the neonatal period (PND 7) and brains were extracted and frozen as described above.

4.5. *Molecular procedures*

4.5.1. *Study 1A and B: Immunohistochemical studies*

Prior to immunohistochemical procedures, brains were fixed in a 4% paraformaldehyde solution. The brains were dehydrated through ascending graded concentrations (10%, 20%, and 30%) of sucrose in 4% paraformaldehyde. The brains were then sectioned (25 μm) using a cryostat (Leica HM 500 OM, Microm International GmbH, Walldorf, Germany). The brain was sectioned throughout all the areas of interest and consecutive sections were mounted onto separate slides, such that each brain area was able to be stained by all antibodies. Immunohistochemical analysis was performed according to Mortensen and Larsson (2001) and Mehra et al. (2005). Tissue sections (25 μm) were incubated with 5% normal goat serum (Antibodies Incorporated, Davis, CA) in sPBS for 10 min to block non-specific binding. Tissues were then exposed to specific monoclonal antibodies for the target proteins [anti-Neu-N (Chemicon, Temecula, CA); anti-synaptophysin (Sigma Chemicals, St. Louis, MO); anti-S-100 (Sigma Chemicals, St. Louis, MO); anti-NCAM (Sigma Chemicals, St. Louis, MO); anti-GAP-43 (Sigma Chemicals, St. Louis, MO); anti-BDNF (rabbit polyclonal, Chemicon, Temecula, CA)]. Exposure to TRITC coupled anti-mouse IgG was used for Neu-N, synaptophysin, GAP-43, NCAM, and S-100 antibodies and FITC coupled anti-rabbit IgG for the BDNF antibody followed. The slides were washed with sPBS buffer and mounted using immu-mount mounting media (Thermo Electron Corporation, Pittsburgh, PA).

4.5.2. *Study 1C: Western blot analysis*

Western blot analysis was carried out as described by Miyake et al. (2002). Samples from each brain site were collected by puncturing the area of 25 μm sections from MR-CONTROL, MR-SHAM, AR-MIN, and AR-MAX brains. Samples were taken from four animals from the same group and pooled together in 15 μl of 10 mM phosphate buffer. Samples were sonicated for 5 s at setting two. Proteins were assayed using Pierce's BCA protein assay reagent (PIERCE, Rockford, IL, USA). SDS-sample buffer (5 ml, 4× concentration) was added to each sample.

Five micrograms of protein from each sample were loaded in each lane and separated by SDS-polyacrylamide gel electrophoresis in BioRad mini gels (BioRad, Hercules, CA). Separated proteins were transferred to PVDF membranes using a BioRad mini transblot apparatus. Membranes were incubated with monoclonal anti-synaptophysin, anti-S-100, anti-NCAM, and anti-GAP-43 antibody followed by HRP coupled anti-mouse IgG. For BDNF the same procedure was applied, but the primary antibody was anti-BDNF polyclonal (rabbit polyclonal,

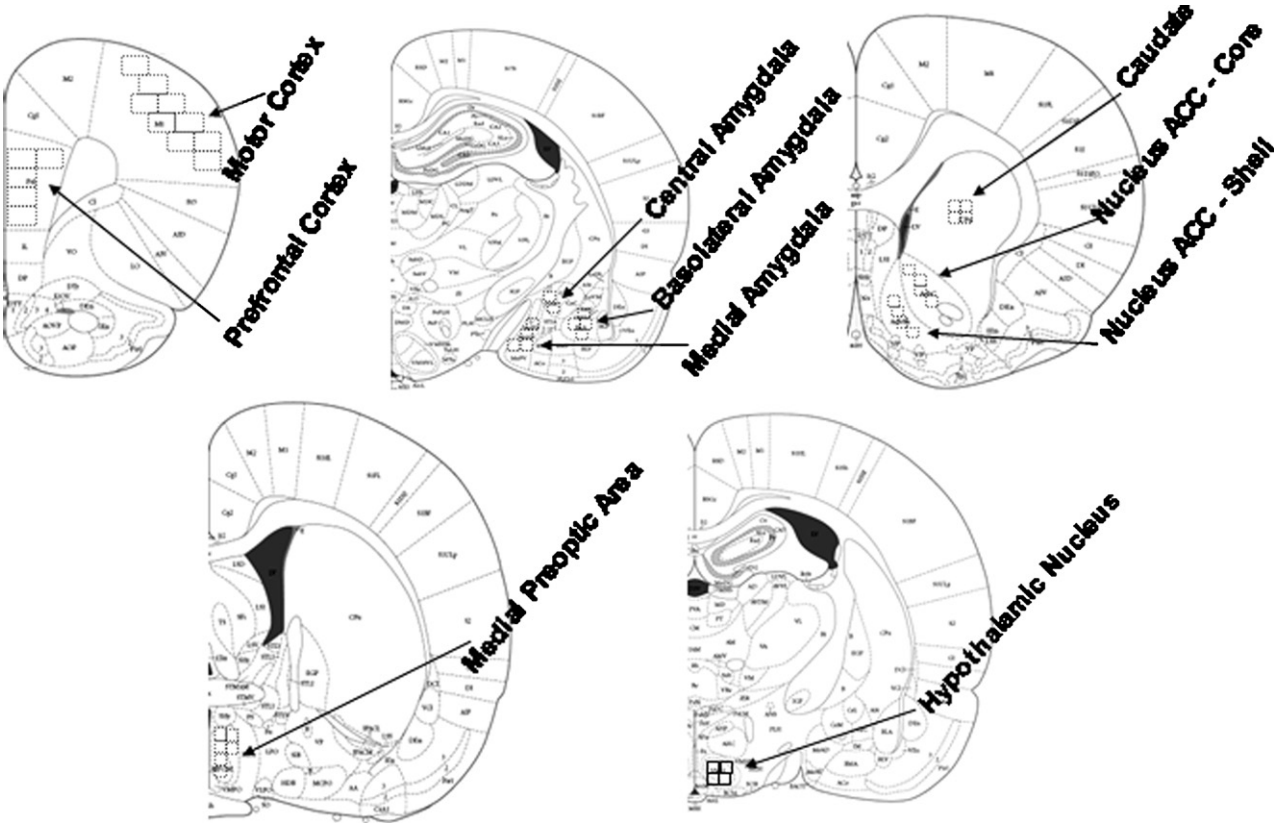

Fig. 8 – Schematic diagrams of the brain areas where pictures were taken for analysis.

Chemicon, Temecula, CA) and the secondary antibody was HRP coupled anti-rabbit IgG (Sigma Chemicals, St. Louis, MO). The immunoreactivity was visualized by ECL technique according to the manufacturer's instructions (GE Healthcare Bio-Sciences Corp., Cardiff, Wales) using a phospho-imager.

4.5.3. *Study 2: TUNEL staining for apoptosis*

Apoptosis was identified by labeling the DNA 3′-OH nick-ends using a variant of TUNEL staining of 25 μm brain sections. Staining for cell death was carried out according to the manufacturer's instructions using the materials provided in the kit (Roche Diagnostics). Briefly, rats (PND 7) were decapitated. The brains were immediately extracted and fixed in 4% paraformaldehyde and ultimately transferred to a 30% sucrose solution in 4% paraformaldehyde containing PBS. Coronal sections (25 μm) were cut using a cryostat and transferred onto superfrost plus slides (Fisher Scientific, Canada) and stored at −80 °C. Brain cryosections were then treated with ice cold 0.1% Triton X100 in Tris buffered saline (TBS, pH 7.4) for 2-min followed by 5 washes with TBS. The sections were then incubated in a humidified chamber at 37 °C for 1-h in the presence of terminal deoxytransferase (TdT) and FITC-labeled nucleotides (enzyme-substrate reagent from kit). After washing 5 times with (2 min each wash) TBS, the slides were mounted using immu-mount mounting media (Thermo Shandon, Pittsburgh, PA, USA). The positive cells in each region were detected under the fluorescence microscope using imaging procedures described below, but adjusted for PND 7 brains.

4.6. *Image analysis*

A number of sections across the anterior–posterior (A–P) sectioning plane were collected from the various brain sites. Juvenile brains were assessed for anti-Neu-N, anti-synaptophysin, anti-S-100, anti-NCAM, and anti-GAP-43. Adult brains were assessed for the same antibodies with the exception of anti-GAP-43, which was substituted with anti-BDNF. All slides were coded for group identification such that experimenters completing the microscopy, imaging, and quantification were blind to the groups and conditions. For inter-observer reliability, the same sections for each brain area for 10 juvenile brains and 10 adult brains were quantified by two independent experimenters. For intra-observer reliability, 1/2 of all sections were quantified two times by the same experimenter. In both cases reliabilities (r) exceeded 0.90.

Immunoreactivity was visualized under an immunofluorescence microscope (OLYMPUS BX60, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) software. Proper filters for TRICT were used with a 10× objective (Neu-N staining) or 20× objective (synaptophysin, S-100, NCAM, and GAP-43 staining, TUNEL). Different brain sites were identified on the basis of surrounding landmarks and pictures were taken from identical sites based on these landmarks. Fig. 8 shows a schematic diagram of the sites where pictures were taken depending on the landmarks in the brain sections. For analysis, four to nine pictures were taken of a particular brain site from each section, depending on the area of interest; and 8–18 fields per animal for each area and antibody (expressed as

number of cells or number of pixels/mm^2). For most brain sites, one side of the brain was quantified using three A–P sections for each site and the average of the 3 sections was taken.[2] Pictures were taken using a 20× objective at a fixed exposure time for each type of staining. The exposure time was set on the basis of the highest signal to noise ratio for that particular antibody staining under the conditions of our microscope and imaging software factory setting (e.g. 8 s for Neu-N staining; 6 s for synaptophysin, NCAM, GAP-43, and S-100 staining; 12 s for BDNF staining). For Neu-N staining, the background for all pictures was set to an identical intensity and the number of nuclei was counted using Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) software using a size-cut off value of 200. Neu-N staining was expressed as the number of neuronal nuclei per mm^2. Backgrounds for all pictures were set to identical settings using the digital control of the Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) software (pixels ranged from 0 to 3) for synaptophysin, S-100, NCAM, BDNF, and GAP-43. Staining intensity was measured using Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) software and expressed as pixel per mm^2.

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[2] For some sites (MPOA and AMY) a single section was imaged and quantified. However, when we compared sites in which 3 A–P sections were analyzed (mPFC, MC, VMH, NAC, NAS, and Caudate) and compared the average of the three sections with the value of the middle section, we found very little difference in the densities indicating that the value of the one section was a good representation of the A–P sectioning plane of the quantified site.

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