Plasticity in the Maternal Circuit: Effects of Pup Exposure and Retention Interval on Astrocyte Numbers in Primiparous and Multiparous Animals.

by

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A thesis submitted in conformity with the requirements for the degree of Master of Arts
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0-612-29187-1
Table of Contents

1. Abstract iii
2. Introduction 4
   i) Hormonal Onset of Maternal Behavior 4
   ii) Postpartum Maintenance of Maternal Behavior 6
       a) Temporal Parameters of Maternal Learning 6
       b) Sensory Parameters of Maternal Learning 7
   iii) Nature of Maternal Learning 8
   iv) Brain Areas Involved in Maternal Behavior 9
   v) Molecular Markers of Brain Activity in the Context of Maternal Behavior 10
   vi) A Role For Astrocytes in Learning 13
   vii) Astrocyte Involvement in Learning 14
   viii) Astrocyte Involvement in Maternal Behavior 16
   ix) Aims of the Present Study 17
2. Methods 17
   i) Subjects 17
   ii) Group Design 18
   iii) Procedures 18
   iv) Immunohistochemistry and Quantification 20
3) Results 21
   i) Maternal Behavior 21
   ii) Astrocyte Number 22
   iii) Correlations Between Behavior and Astrocyte Cell Counts 23
4) Discussion 24
5) References 35
6) Figure Legends 43
7) Figures 45
Experience with pups postpartum enhances the expression of maternal behavior up to a month later. These long-term behavioral changes are accompanied by long-term changes in immunohistochemical expression of neuronal and glial proteins, specifically Fos, Protein Kinase C (PKC), and Glial Fibrillary Acidic Protein (GFAP) in the medial preoptic area.

The goal of the present study was to examine astrocytic changes (as assessed by Glial Fibrillary Acidic Protein) following varying amounts of maternal experience and over a longer time-frame. Primiparous and multiparous animals (2x pregnant), were given a brief exposure (either 2 or 24 hours) to 1-3 day old rat pups the day following parturition, or were given a similar control manipulation. Animals were then sacrificed and subjected to immunohistochemistry either 1 day, 5 days or 10 days following postpartum pup exposure.

A main effect for parity in favor of multiparous animals was seen in the MPOA. An opposite effect for parity was found in the medial and lateral habenula, as well as the medial amygdala (MEA), with primiparous animals having higher numbers of astrocytes than multiparous animals. Parity differences in the MPOA were slight at one day after exposure/manipulation, but were significantly different at day five. At day ten, parity differences were seen, but were dependent upon pup exposure. In contrast, in the MEA and habenula nuclei, parity differences were most substantial at day one.

The results suggest that primiparous and multiparous animals differ not only in what they learn, as evidenced from the dissociation between MPOA and MEA/habenula brain regions, but also differ in terms of when learning takes place.
Introduction

Maternal behavior can be conceptualized as consisting of three separate components, initiation, maintenance and retention (Bridges, 1990). Initiation refers to the change in a new mother from a state of unresponsiveness or avoidance towards young, typical of maternally inexperienced dams, to a state where the animal will readily display pup directed behaviors. Once initiated, enhancements of maternal behavior must also be maintained for a period long enough for the dam to provide adequate care for her young. Finally, retention refers to the tendency for animals which have experienced prior pup interactions, and subsequent initiation of maternal behavior, to respond maternally to young in the future in a quicker and more efficient manner than animals inexperienced with young.

Hormonal Onset of Maternal Behavior

The mechanisms responsible for the initiation of maternal behavior are reasonably well understood, and appear to be primarily hormonal. If virgin animals (which are normally unresponsive to pups) are given hormone treatments which mimic the pattern of hormone activity around the time of parturition they will show an increase in responsiveness to rat pups similar to that seen in the pregnant animal (Moltz, et al, 1970), as well as a general reduction in fear responses to stimuli scented with the odor of pups (Fleming, et al, 1989). Early support for the notion of hormones as facilitators of maternal behavior came from an experiment conducted by Terkel and Rosenblatt (1968) in which blood plasma was removed from new mother rats and injected into inexperienced virgin rats. Virgin rats given such treatments demonstrated significantly
reduced latencies to become maternal. These researchers conducted a similar experiment in which virgin animals were connected via a catheter to new mother rats (Terkel and Rosenblatt, 1972). The catheter allowed blood to flow between the two rats such that, over time, animals would share a common blood constitution. The results showed that blood delivered from a newly parturient rat caused a shortening of the latency for virgin animals to respond maternally. Later studies investigating the nature of hormone changes important for the induction of maternal behavior have pointed to a role for estrogen (Rosenblatt and Siegel, 1975), especially in association with declining progesterone levels (Bridges, 1990). Other hormones of importance include prolactin (Bridges, et al, 1985; Bridges and Mann, 1994) and oxytocin (Pedersen, et al, 1982; Pedersen, et al, 1985; Fahrbach, et al 1984; Fahrbach, et al, 1985; Insel, 1990).

Interestingly, while hormones appear to be sufficient for the rapid initiation of maternal behavior, it remains questionable as to whether they are necessary for the behavior to be expressed. With sufficient exposure to pups (approx 4 days: Rosenblatt, 1967), virgin rats will move from a state of active avoidance of pups to one of active interaction (Rosenblatt, 1967), although it is not known for certain that such sensitization is not, at least in part, hormonally mediated, although such sensitization has been shown to occur even in ovariectomized animals lacking hormones (Rosenblatt, 1967). Additionally, pre-pubescent rats appear to show a brief period, between 20 and 25 days of age, where they will readily display many of the component behaviors associated with maternal behavior (Bridges, et al, 1974). In such animals, pubertal hormonal changes have not yet occurred.
Postpartum Maintenance of Maternal Behavior

While hormones, either exogenous or endogenous, have been shown to influence the initiation of maternal behavior, maintenance and longer term retention of maternal behavior appears to require interaction with pups during times of enhanced hormonal changes. This finding was established in early studies which compared the behavior of animals allowed postpartum pup contact following parturition with similar postpartum animals prevented from engaging in such contact. These studies clearly demonstrate that parturition, in and of itself, is not sufficient to allow for long term changes in maternal responsiveness (Slotnick, et al, 1973; Bridges, 1975; Bridges, 1977). Postpartum animals which were prevented from interacting with pups were found to be no more responsive to pups 25 days following parturition than inexperienced virgin animals. Only when animals were given postpartum pup contact did they show any retention of maternal responsiveness at retest (Bridges, 1977).

Temporal Parameters of Maternal Learning

Apparently, very little postpartum pup contact is necessary to produce long term behavioral changes in new dams. Animals given either one, four or nine days of pup contact following the establishment, via continuous pup exposure, of full maternal behavior appear to be equally responsive to pups upon retest 25 days later (Cohen and Bridges, 1981), suggesting that pup exposures of as little as one day are sufficient to produce long term changes in behavior. In a further study investigating this issue, Orpen and Fleming (1987) gave animals even shorter amounts of pup exposure, ranging from 15 minutes, 30 minutes and twenty-four hours, on the day following cesarean removal of pups. Ten days later, animals were retested. The animals given 30
minutes of pup contact were found to be as responsive to pups (in terms of latency to become maternal) ten days later as were those animals given 24 hours pup contact. Then, ten or thirty days later, animals were tested for responsiveness to rat pups. The results demonstrated that all pup exposed animals, regardless of the amount of pup exposure received, were equally maternally responsive at retest ten days following initial exposure. Thus, post partum experience with pups, even with short term exposure, produces changes in maternal responsiveness which last as long as ten days, and which is not seen in animals who merely experience parturition without subsequent pup exposure. However, the expression of learning past this point appears to be somewhat less clear.

Sensory Parameters of Maternal Learning

A number of somatosensory features inherent in the initial pup exposure appear to be crucial for the long term retention of maternal behavior. For example, when mother animals were deprived of tactile input, by blocking somatosensory input to the ventral and perioral areas during initial postpartum interactions with pups, they are found to be much less responsive to pups ten days later than were animals which had been given such stimulation (Orpen and Fleming, 1987; Morgan, et al, 1992). Apparently, tactile stimulation is necessary for long term enhancement of maternal responding. However, it should be noted that tactile deprivation does not completely block maternal learning, since animals exposed to pups from a distance will still show enhanced responsiveness when compared to completely deprived animals (Orpen & Fleming, 1987).

In contrast, other sensory input appears to be less important for the maternal
experience effect. In the case of olfactory stimulation, animals given pup exposure after undergoing olfactory denervation fail to show great deficits in maternal response upon later re-exposure to pups (Fleming, et al, 1992), suggesting that olfactory input is not necessary for the maintenance of experience enhanced maternal responsiveness. Finally, auditory, thermal and visual cues present during the mother's initial interaction with pups are likely retained by the mother and, thus, contribute to the maternal experience effect. However, it is unlikely that these cues, in and of themselves, are sufficient to produce long term changes in maternal responding.

Nature of Maternal Learning

What is it about postpartum pup experience that enhances long term maternal responsiveness? More precisely, what is it that dams learn during pup exposure that allow for long-term changes in maternal responsivity? One important change which occurs in the postpartum animal, and which separates the postpartum animal from a similar nulliparous animal, is the change (perhaps hormonally stimulated) in the how the dam perceives pups and pup related stimuli. Further, unlike nulliparous animals, postpartum animals appear to view rat pups as highly reinforcing stimuli, and will develop a conditioned place preference to an experimental chamber in which they have received pup contact (Fleming, Korsmitt and Deller, 1994). Postpartum dams will also readily learn to press a bar to gain access to rat pups in a standard operant chamber, an effect which seems to depend, at least in part, on similar neural substrates as those used in traditional reward paradigms (Lee, et al, in prep).
Similarly, while initial hormone priming may increase the willingness to learn the particular behaviors associated with maternal behavior, the actual performance of these behaviors, which is dependent upon pup contact, may be necessary for any long term learning to take place. Thus, when a postpartum animal is prevented from engaging in pup contact, it is denied the opportunity to learn the particular behaviors associated with maternal behavior. Not surprisingly, then, if such an animal is later tested for maternal responsiveness, it will show little ability to perform such behaviors.

Thus, long-term changes in maternal behavior could be the result of two types of learning. Prior experience with pups could function to change for the long-term the motivational state of the animal, or more precisely, change how the animal perceives young. Conversely, animals may also learn to perform maternal behaviors more efficiently. To some extent, this distinction is similar to the distinction, often made in cognitive psychology, between episodic and procedural memory. Changes in the actual performance of maternal behavior (more efficient retrieval, nest-building, etc.) would reflect this procedural type learning, while changes in overall responsiveness may reflect a more episodic type of learning. Perhaps, this episodic learning involves learning that rat pups are distinct stimuli with a distinct set of behaviors attached to them.

Brain Areas Involved in Maternal Behavior

To date, a number of brain sites appear to be important for maternal behavior. Lesion studies have reliably demonstrated deficits in the display of maternal behavior, especially retrieval, following destruction of the MPOA in adult rats (Numan, et al, 1988; Numan, et al, 1990, Numan and Numan, 1991), and also in juvenile rats (approx 30
days of age) induced to become maternal through continuous exposure to rat pups (Natterer and Fleming, in prep). Electrical stimulation of the MPOA has been demonstrated to decrease the amount of time needed for virgin animals to display maternal behavior (Morgan, Watchus and Fleming, 1996). Further evidence for a role of the MPOA in maternal behavior has been suggested through studies involving immunohistochemistry (C-fos, Walsh, et al, 1996; Fleming and Korsmit, 1996; Numan and Numan, 1994; PKC & GFAP, Fleming et al, 1996).

Amygdaloid nuclei have also been implicated in maternal behavior. For example, lesions of the medial amygdala appear to facilitate the occurrence of maternal behavior (Fleming, Vaccarino and Luebke, 1980; Numan, et al, 1991). Likely, the amygdala is important for sensory learning which occurs during maternal behavior, and appears to be central in the production of initial fear reactions of new mothers to rat pups.

Other areas of importance to maternal behavior include the nucleus accumbens (Fleming and Lee, in prep), the lateral habenula (Matthews-Felton, et al, 1995), and the parietal cortex (Xerri, et al, 1994; Fleming and Korsmit, 1996; Fleming, et al, 1996). Interestingly, dramatic plastic changes occur in the parietal cortex following maternal behavior, with areas of somatosensory cortex responsible for detecting ventral sensation rapidly expanding during pup contact, often into regions of the somatosensory cortex formerly representing other areas of the body (Xerri, et al, 1994).

Molecular Markers of Brain Activity in the Context of Maternal Learning
In recent years, focus in maternal behavior research has increasingly shifted towards the understanding of the underlying molecular substrates of maternal behavior. In part,
this has involved the search for the underlying neural and cellular basis of maternal learning, as well as the neurochemical changes important for the expression and retention of maternal behavior. To date, a number of proteins have been identified as being altered during maternal behavior.

Cyclohexamide is a substance known to block the synthesis of proteins, and appears to play an important role in the consolidation of maternal experience. Specifically, I.C.V. administration of Cyclohexamide following a brief maternal experience was found to disrupt responsiveness to pups upon retest ten days following initial exposure (Fleming, et al, 1993). Importantly, the disruptive effects of Cyclohexamide were less salient in those animals given the drug prior to initial exposure, suggesting that Cyclohexamide is important for the 'consolidation' but not the 'acquisition' of maternal memories.

We have obtained evidence suggesting that brief maternal experiences (contact and interaction with rat pups) can lead to long term functional changes in the brain. C-fos, a proto-oncogene, is known to increase production of its protein product (Fos) in response to neural activity. Evidence suggests that rats which had been given a brief four hour exposure to pups one day following parturition, as compared to rats who had not been exposed to pups, showed higher levels of Fos-like immunoreactivity in certain brain areas following re-exposure to pups ten days later (Fleming and Korsmit, 1996). Areas showing significant c-fos expression included the medial preoptic area of the hypothalamus (MPOA), basolateral amygdala, the cingulate/prefrontal cortex and the parietal cortex.

Maternal behaviour induced increases in Fos immunoreactivity have been
demonstrated in other experiments and in other labs. Specifically, increases in Fos expression have been found in the MPOA as well as the ventral bed nucleus of the stria terminalis (BNST) following pup exposure which allowed full behavioural interactions with pups (Numan & Numan, 1995). Similar increases in Fos immunoreactivity have been shown following maternal experience in mice (Calamandrei & Kaverne, 1994). Interestingly, in this study, distal exposure to pups (pups were presented in a manner which prevented direct pup contact) was found to cause increases in Fos immunoreactivity in peripheral sensory areas (anterior olfactory nucleus), but not in more central areas such as the MPOA (Calamandrei & Kaverne, 1994).

Proteins upstream in the neurochemical cascade responsible for Fos production also appear to be involved in maternal learning. Protein Kinase C is a protein responsible for phosphorylation of protein substrates, many of which are vital for normal cellular functioning. In a recent experiment, we have shown that PKC undergoes an increase following maternal experience. Specifically, animals were given either a 2hr pup exposure on one day, or consecutive 2hr exposures over a three day period. Animals were then sacrificed either immediately after exposure, or three days following exposure, in the case of animals given the single 2hr exposures, or immediately after exposure, in the case of the consecutive 3 day exposed animals. In comparison to animals given no exposure, all pup exposed animals showed increased levels of PKC immunohistochemical staining in both the MPOA and parietal cortex (Fleming, et al, 1996). Interestingly, neither the length of exposure, or amount of time interval between exposure and sacrifice had significant effects upon the level of PKC staining. Apparently, PKC levels increase immediately following exposure and are maintained for
A period of time long after exposure.

A Role for Astrocytes in Learning

Astrocytes possess a number of features which make them well suited to play a role in learning. For example, astrocytes appear to express receptors for most neuroactive substances. In terms of the so called 'classical' neurotransmitters, astrocytes have been demonstrated to possess adrenergic receptors (Shao, et al, 1993; Bowman & Kimelberg, 1987), amino acid receptors (Cornell-Bell & Finkbeiner, 1991), and GABA receptors (Bormann & Kettenmann, 1988).

Astrocyte receptors for peptide and hormonal substances are also quite robust. These receptors include an estrogen induced progesterone receptor (Jung-Testas, et al, 1991), oxytocin (Di Scala-Guenot, et al, 1992), neuropeptide Y (Gimpl, et al, 1993) and angiotensin (Sumners, et al, 1991; Raizada, et al, 1993). More importantly, perhaps, many of these substances have been shown to have extensive effects on astroglial proliferation and morphology, especially estrogen (Luquin, et al, 1993; Tranque, et al, 1987) and oxytocin/vasopressin (Lucas & Salm, 1995). Similar results have been shown with both thyroid hormones (Andres-Barquin, et al, 1993) and testosterone (Day, et al, 1995; Day, et al, 1993; Garcia-Segura, et al, 1988), suggesting that astroglia may contain receptors specific for these substances.

Astrocytes have also been shown to produce and secrete a number of neuroactive substances, including amino acid compounds such as glutamate, aspartate and GABA (Dutton, 1993; Martin, 1992), neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, and fibroblast growth factor (Rudge, 1993), as well as
precursor substances for most neurotransmitter and hormone substances (Martin, 1992).

While the existence of receptors, and the ability to secrete neuroactive substances, suggests that astrocytes have an adequate ability to monitor and respond to neural events in a manner traditionally ascribed to neurons, astrocytes also appear to possess more unique means of influencing brain activity. For example, astrocytes can modulate extracellular pH levels (Ransom, 1992). Many studies have demonstrated that extracellular pH levels can produce significant effects upon neural activity. Lowering of pH, for example, decreases the ability of Mg2+ to induce seizure activity in brain slices (Velisek, et al, 1994). In general, acidification is thought to greatly reduce neuronal activation (Ransom, 1992). Interestingly, depolarization of glial cells has been shown to cause both intracellular alkalization and extracellular acidification (Ransom, 1992), suggesting that, through pH regulation, astrocytes can significantly effect neuronal activity. In a similar manner, it has been suggested that astrocytes may play a role in the regulation of extracellular ionic concentrations (Kimelberg, et al, 1993).

Astrocyte Involvement in Learning

Some evidence exists for the participation of astrocytes in learning within several experimental paradigms. For example, cellular models of learning, such as kindling and long-term potentiation, have been used to demonstrate changes in astrocytes in response to electrical stimulation. Kindling of the hippocampus has been demonstrated to result in increases in GFAP in areas receiving hippocampal input, such as the pyriform cortex and amygdala, as well as in the hippocampus itself (Hansen, et al, 1990). Amygdaloid kindling results in increased GFAP in the hippocampus, pyriform

Several behavioral paradigms have also been used to demonstrate a potential role for astrocytes in learning. A series of experiments have examined astrocyte changes in response to environmental enrichment. In a standard experiment, animals are exposed to either an isolated (left alone in cage), social (placed in cage with age mate) or enriched environment. Typically, animals exposed to environmental enrichment show astrocytic changes not found in animals given either isolated or social exposures. Astrocyte changes have included astrocyte size and number of processes (Sirevaag, et al, 1988), increased surface density of processes (Sirevaag, et al, 1991) increased number of astrocytic nuclei (Sirevaag, et al, 1987), and increased astrocyte volume per neuronal cell (Anderson, et al, 1994).

Developmentally, astrocytes appear to be crucial in the creation of some neural organizations. For example, researchers have found that astrocytes, as measured by GFAP immunohistochemistry, undergo changes in structural organization in response to environmental stimulation. In the lateral geniculate nucleus of the thalamus, glial cells appear to develop, in response to environmental simulation, a layered organization, similar to the eye specific neuronal layering characteristic of the visual thalamus (Hutchins & Casagrande, 1990). Similarly, in the area of somatosensory cortex known to be involved in the representation of vibrissa, and which has been shown to possess a distinct neuronal topography, astrocytes have been demonstrated to develop a similar topography (Cooper & Steindler, 1989; Steindler, et al, 1989). What is important in both cases, is that the particular glial organizations appear to develop prior to the
development of their corresponding neuronal counterparts, and seem to disappear once the neuronal structures have been created (Hutchins & Casagrande, 1990; Cooper & Steindler, 1989; Steindler, et al, 1989). Such results suggest that glia are necessary for the development of such structures, at least during development.

Astrocyte Involvement in Maternal Behavior

A number of researchers have suggested a role for astrocytes in neuronal restructuring following parturition and lactation. Specifically, it has been demonstrated that glial processes in the supraoptic nucleus (SON) (Hatton and Tweedle, 1982) and paraventricular nucleus (PVN) (Theodosis and Poulain, 1989), which normally separate neurons in these areas, withdraw during lactation, resulting in direct neuronal-neuronal contacts. Interestingly, glial withdrawal tends to occur primarily in relationship to oxytocinergic neurons, and not with vasopressin secreting cells (Theodosis, et al, 1986). These changes appear to occur shortly before the onset of parturition, and remain for about a month, after which point they return to levels similar to that seen in virgin animals (Montagnese, et al, 1987). Further, the changes appear to be dependent upon exposure to pups. Animals which undergo parturition, but which are prevented from gaining postpartum contact with their pups do not appear to show increases in neuronal juxtapositions (Montagnese, et al, 1987).

In our lab, we have found evidence that maternal experiences can lead to substantial changes in the expression of GFAP in brain areas (MPOA and parietal cortices) known to be involved in maternal learning (Fleming, et al, 1996). Specifically,
animals examined five days after their second pregnancy (multiparous) were found to have higher levels of GFAP (as reflected in number of GFAP positive astrocytes) than did animals examined five days following a first pregnancy (primiparous). Both primiparous and multiparous partum animals displayed higher levels of GFAP stained astrocytes than did similar non-partum animals.

Aims of Present Study

The goal of the present study is to investigate how astrocytes change in response to maternal experience under a wide range of temporal and experiential conditions. To date, studies in our lab have focussed solely on astrocytic changes occurring around day five postpartum. The rationale for such a procedure has been based on the fact that, by day five postpartum, hormonal elevations associated with parturition decline to prepartum levels. While this is optimal for dissociating the effects of learning on astrocytes from the effects of hormones, it does not allow for a full appreciation of how astrocytes change over the duration of postpartum learning. Thus, in the present study, animals were either given 2, 24 or no pup exposure, and then were sacrificed either 1 day, 5 days or 10 days following exposure.

METHODS

Subjects.

N female rats, of the Sprague-Dawley type, ranging from 60 to 100 days of age, obtained from Charles Rivers Farms in Quebec, were used. Subjects were housed in opaque cages 45cm long X 23cm wide X 15.5cm deep, located within the same room.
Cages were covered with a steel wire top, designed in such a way that the subjects had ad lib access to food (Purina rat chow) and water. Subjects were kept on a 12 hour light cycle, with lights on from 8am to 8pm. The room temperature was maintained at 22 degrees Celsius.

Group Design.

In total, 18 groups were created. Groups differed in terms of prior parity (primiparous versus multiparous), day sacrificed (1 day, 5 days or 10 days following exposure/manipulation) and pup exposure (non-exposed, 2 hr exposure and 24 hr exposure). Within each parity condition (multiparous and primiparous), groups were balanced so that all possible combinations of the two remaining variables (day sacrificed and pup exposure) were obtained (i.e. 1 day non-exposed, 2 hr exposed, 24 hr exposed; 5 day non-exposed, 2 hr exposed, 24 hr exposed; 10 day non-exposed, 2 hr exposed, 24 hr exposed), such that a completely crossed 3 variable design was produced (see figure 1).

Procedures.

For impregnation, animals were housed with a stud male for a period of 5 days. On day 21 following mating, pregnant animals were monitored at 15 minute intervals between 10:00 and 21:00. During each interval, any newborn pups were removed to prevent the dam from gaining experience with pups. Following termination of parturition, animals were removed from the birthing room, placed in fresh cages and taken to a room containing pup deprived animals.

On the day following parturition, animals to be given pup exposures were habituated to a fresh cage and a small amount of shredded paper towel for a period of
approximately one hour. Following this, 6 rat pups (1 to 3 days of age) were placed in the cage with the dam, and the behavior of the dam was observed for an eight minute period. During observations, behaviors were recorded for each five minute interval within the eight minute period. Behaviors which were recorded included: approach, which was defined as any instance wherein the mother rat moved from an area distal to her pups to one in close proximity to them; withdrawal, which was the opposite of approach; grooming, as indicated by any occurrence of self-cleaning behaviour exhibited by the mother, such as licking or biting herself, etc.; retrieval, wherein the mother rat grasped a pup within her teeth and moved it from one area to another; nest-building, which was defined as any occurrence of paper shredding, moving of paper, digging at the bedding lining the bottom of the cage or the building of mounds with the bedding or paper towel; pup licking, where the mother was seen placing her tongue on the pup; anogenital licking, which was defined as any instance where the mother was observed licking a pup in the region of the genitals; crouching posture, wherein the mother laid over the pups, adopting a crouching position over them.

Subjects were then left with pups for the appropriate period of time (2 or 24 hours) with spot-checks being performed, in the case of 2 hour exposed animals, every fifteen minutes following termination of the 8 minute observation period, or, in the case of the 24 hour exposed animals every two hours for the first four hours of exposure following termination of the initial observation period. Following the termination of exposure, animals were placed in a fresh cage and moved back into the pup deprived room wherein they remained until sacrificed. All non-exposed animals underwent identical manipulations, with the exception of being exposed to rat pups. Exposures
were carried out between 14:00 and 20:00 hours. Animals which failed to meet a predetermined criterion for maternal behavior (retrieval of pups to a common nest site and licking of pups) were excluded from the study.

Immunohistochemistry and Quantification.
Following the appropriate amount of time (i.e. 1 day, 5 days or 10 days), animals were sacrificed via a lethal dose (.7cc) of sodium pentobarbital (Somnotol, MTC Pharmaceuticals, MO). Subjects were then perfused intracardially with a saline solution for two minutes, followed by a 4% paraformaldehyde solution for six minutes. After perfusion, brains were removed and post-fixed in the paraformaldehyde solution for approximately 12 hours, after which they were moved to a sucrose/paraformaldehyde (30% sucrose) solution were they remained for approximately 48 hours. Brains were then frozen (-18 degrees celsius), sliced on a sliding microtome (40 um slices) and placed within a PBSx solution (0.01 M phosphate-buffer saline with 0.3% Triton-X) (Triton-X obtained from Sigma).

For immunohistochemistry, brain slices were first incubated for 48 hours within a solution containing mouse monoclonal anti-GFAP antibody (Boehringer Mannheim, Canada), in a 1:400 (GFAP) dilution. Following this, slices were washed three times (10 minutes each) in the PBSx solution, before being placed in a secondary antibody solution (1:1500), consisting of 1% goat serum (Dimension Labs, Mississauga, Ontario, Canada), PBSx and anti-mouse immunoglobulin, for a period of two hours. Slices then underwent three PBSx washes after which they were placed in a standard avitin-biotin complex (Vector Labs, Burlington, Canada) for one hour. After three PBSx washes,
slices were immersed in a phosphate buffered saline solution containing diaminobenzadine (DAB) (Sigma, Canada) for 15 minutes. To visualize staining, a glucose oxidase solution was added to the DAB solution for 20 minutes. Immunohistochemistry was completed with two additional 10 minute PBSx washes. Following immunohistochemistry, slices were mounted on gel-coated slides and coverslipped.

Astrocyte cell counts were carried out with a standard light microscope (10 X 15 magnification), equipped with a 1 mm squared grid reticule (Baxter, Canada).

At .26 mm posterior to bregma, astrocyte counts were taken for the MPOA, parietal cortices (PAR1 and PAR2) and ventral bed nucleus of the stria terminalis (VBNST). The MPOA region was subdivided into its nuclear constituents (AMPO, AVPO and MPA).

Parietal astrocyte counts were taken in two areas (PAR1 and PAR2), with PAR2 being immediately ventral to PAR2 (refer to figure 2 for location of brain sites at the -.26 level). Finally, at 2.8 mm posterior to bregma, cell counts were taken for the basolateral amygdala (BLA), medial amygdala (MEA), cortical amygdala (ACO and PICO), dorsal hippocampus (CA1), ventromedial hypothalamus (VMH) and the medial and lateral habenula (refer to figure three for the location of these brain sites). Each brain site was counted bilaterally.

Results

Maternal Behavior

In total, 107 animals participated in the study (9 animals were excluded because they
failed to meet the maternal criterion, and an additional animal was excluded because it was found to have cell count numbers in all of the brain sites examined greater than two standard deviations below the mean average for its group). A one way anova was conducted which compared maternal behavior between primiparous and multiparous animals, both during the initial eight minute observation as well as during the subsequent spot-checks. During the eight minute observation, multiparous animals were found to be significantly more likely to engage in both genital licking (F(1,72)=25.65, p<.000) and pup retrieval (F(1,72)=4.09, p<.047). Alternatively, primiparous animals were more likely to engage in pup sniffing (F(1,72)=2.5, p<.008). Marginal differences were seen for nest building, with multiparous animals showing slightly higher levels of this behavior than did primiparous animals (F(1,72)=3.09, p<.083).

Astrocyte Numbers

A series of two (parity) x two (exposure) analyses were conducted which compared parity and exposure effects on astrocyte numbers in the MPOA regions within each day sacrificed condition (see figure 4 for details). At day one, no differences were seen between primiparous and multiparous animals, nor between any of the exposure conditions. A significant effect for parity was found at day five, with multiparous animals showing significantly higher numbers of astrocytes in the AMPO (F(1,27)=6.98, p<.014), MPA (F(1,27)=5.84, p<.023) and MPOA TOTAL (F(1,27)=5.41, p<.028). No exposure effects were found. At day ten, significant two way interactions were found between parity and exposure in the AVPO (F(2,34)=6.17, p<.005), MPA (F(2,34)=4.65, p<.016) and MPOA (F(2,34)=5.21, p<.011). No main effects were found for either parity or
A second two way ANOVA was carried out which compared, within each parity condition, the effects of exposure and daysac. For primiparous animals, no effects of either exposure or daysac were observed within any of the MPOA nuclei. In the multiparous group, a significant interaction was found within the AVPO (F(4,45)=2.98, p<.029)

In the remaining brain areas, a similar series of 2 (parity) x 3 (exposure) analyses were conducted which compared parity and exposure effects within each day sacrificed condition. Significant differences between parity conditions were found at day 1 in the MEA (F(1,25)=5.31, p<.30), Lhb (F(1,25)=6.53, p<.017), Mhb (F(1,25)=5.87, p<.023) and parietal cortex (F(1,25)=6.61, p<.016), with, in each case, primiparous animals showing higher numbers of astrocytes than did multiparous animals (see figure 5 for MEA and habenula, figure 6 for remaining areas). At day ten, significant main effects for parity were seen within the MEA (F(1,34)=7.99, p<.008) and in PAR2 (F(1,34)=5.37, p<.02) with primiparous animals showing higher astrocyte numbers than multiparous animals.

A final series of analyses were conducted which compared exposure and day sacrificed within each parity condition. No significant exposure or daysac effects were found for any of these brain sites in either the primiparous or multiparous groups.

Correlations Between Behavior and Astrocyte Cell Counts
A number of correlation coefficients were calculated to assess the relationship between cell count numbers and specific behaviors. The MPOA region was found to be positively correlated with nest building (r=.243, p<.04). MPOA cell counts were negatively
correlated with grooming ($r=-.324, p<.006$), and this relationship appeared to exist for all the brain sites within the MPOA (AMPO ($r=-.327, p<.005$); AVPO ($r=-.254, p<.032$); and MPA ($r=-.305, p<.009$)). To a lesser extent, MPOA astrocyte numbers were also negatively correlated with sniffing pups, although this was only found in the AMPO region ($r=-.250, p<.034$). The MEA area was found to be negatively correlated with lactating posture ($r=-.29 p<.013$), as was the PICO ($r=-.29, p<.013$). Habenula astrocyte numbers were also negatively correlated with genital licking (Lhb ($r=-.331, r=.004$); Mhb ($r=-.335, r=.004$)), and while the Lhb was found to be positively correlated with grooming ($r=.339, p<.003$). A negative correlation was seen between CA1 hippocampal astrocyte numbers and retrieval ($r=-.27, p<.022$), while a negative correlation was seen between BLA cells counts and pup licking ($r=-.227, p<.018$).

**Discussion**

The results of the present study demonstrate that animals which have undergone an earlier parturition and experience with pups (multiparous animals) show significantly higher numbers of astrocytes in the MPOA upon later pregnancies than do first time, primiparous animals. These results are consistent with our previous study in groups sacrificed on day five post partum (Fleming, et al, 1996), and are highly consistent with earlier studies which investigated Fos changes associated with maternal learning, and with what is currently known about the brain areas involved in maternal behavior. For example, the MPOA region is widely known to be involved both with maternal behavior and maternal learning. This has been established both through lesion and
immunohistochemical studies. Thus, maternal learning likely involves increases in neural activity in the MPOA region, and, possibly, changes in astrocytes. Not surprisingly, animals which had undergone a previous experience with pups (multiparous animals) showed higher numbers of astrocytes in this area than did inexperienced (primiparous) animals.

Within the MEA, Lhb and Mhb, however, an opposite relationship was seen, such that animals undergoing their first parturition and experience with pups (primiparous animals) showed higher numbers of astrocytes than did animals which had previously undergone such an experience (multiparous animals). The finding of the opposite effect for parity (primiparous animals having higher levels than multiparous animals) within the medial amygdala (MEA) is also consistent with what is known about the brain areas involved in maternal behavior. It is well known that the amygdaloid nuclei, especially the medial amygdala, are involved in the aversion, in new mothers, towards pups (Fleming, et al, 1980). The typical interpretation of this effect is that, for the new mother, olfactory cues from pups are aversive, and that this aversion is created via amygdaloid activation following pup contact. Destruction of the amygdala, therefore, prevents such aversions from forming, leaving the animal motivated to act maternally. Thus, inexperienced primiparous animals, upon contact with pups, experience increases in amygdala activation, resulting in, or perhaps in part created by, astrocytic increases. A role for the habenula nuclei in maternal behavior has also been suggested. Specifically, lesions of the lateral habenula block the ability of hormones to facilitate the occurrence of maternal behavior (Matthews-Felton, et al, 1995). Interestingly, our previous Fos studies also found significant effects in the lateral
habenula, with inexperienced animals showing higher levels of Fos immunoreactivity than experienced animals (Fleming & Korsmit, 1996). Thus, the lateral habenula appears to be necessary for the induction of maternal behavior, or, more specifically, the change in the animal from a state of unresponsiveness towards young to a state of responsivity. Hence, primiparous animals should be expected to show higher levels of activation in this area than multiparous animals, who have previously undergone this change.

The results of the correlational analyses are also consistent with what is known about maternal behavior, and suggest that changes in levels of astrocyte numbers are related to the occurrence of certain maternal behaviors, but that this relationship depends upon both the behavior being analyzed and the brain site in which the astrocytes reside. In the MPOA, high levels of astrocytes were found to correspond with high levels of pup directed behaviors, such as nest building. In contrast, increased numbers of astrocytes in the MPOA appear to be related to low levels of what could be described as pup avoidance, or fear motivated, behaviors, such as grooming and pup sniffing. Thus, animals with high levels of astrocytes in the MPOA appear to be more likely to view pups as attractive and behave accordingly. In contrast, medial amygdaloid and habenula astrocyte counts showed an opposite relationship, in that high levels of astrocytes appeared to correlate most strongly with decreased levels of pup directed behaviors, in this case anogenital licking. However, astrocyte numbers in these areas did not appear to be positively correlated with pup avoidance behaviors. In fact, the opposite case was seen, with strong negative correlations between cell counts in these areas and both grooming and pup sniffing.
In the MPOA in total, the relationship between prior parity and astrocyte number appears to be dependent both upon the time after parturition at which the changes are assessed and the present pup exposure conditions. In terms of the temporal parameters of astrocyte change, the major parity effect appears to occur at day five, with multiparous animals showing higher levels of astrocytes than primiparous animals. Prior to this, little difference is seen between animals based on parity, with primiparous animals showing astrocyte numbers equivalent to multiparous animals. By day ten, however, parity effects are still found, but appear to become dependent upon pup exposure. Overall, in the MPOA, astrocyte numbers appeared to increase in multiparous animals, from relatively low numbers at day one to increasingly higher levels at days five and ten. Interestingly, primiparous animals seemed to show an opposite trend, with astrocyte numbers being relatively high at day one, then progressively levelling off at days five and ten. If one considers astrocytic changes to be indicative of learning, or of underlying neural changes, then the results of this study suggest that learning in the MPOA occurs in inexperienced (primiparous) animals at different times than experienced (multiparous) animals. Inexperienced animals show the majority of learning (or changes in the potential to learn) early on, shortly after parturition, while multiparous animals appear to show the greatest activation at later times. To some extent this makes sense. In order to be good mothers, inexperienced primiparous animals must learn to approach pups and display appropriate behaviors towards them. Experienced, multiparous animals, however, have likely already learned to do this. For these animals, learning may be more involved in the long term maintenance of responsivity towards pups.
To some extent this is reflected in the parity effects found in the MEA and habenula nuclei. In these areas, strong parity effects were seen in favor of primiparous animals, but these effects were most substantially shown at day one, and, in fact, appeared to dissipate by day five. All of these areas have been implicated in the change in new mothers from a state of avoidance to active involvement with young. Thus, these areas are likely to be involved primarily in new mothers, and primarily at periods shortly after parturition. The fact that astrocyte numbers were decreased in multiparous animals suggests that one consequence of prior experience is a permanent decrease in activation in these areas.

Some effects were seen for pup exposure. However, this appeared to depend upon, first, the parity condition of the animal, and second, the time after exposure that the animal was sacrificed. In general, long term exposure to pups (24 hour) appeared to result in increased numbers of astrocytes in the MPOA TOTAL, AVPO and, most dramatically, in the MPA, in multiparous animals sacrificed at day ten. Animals given such exposure had much greater numbers of astrocytes than animals in the other two conditions. In contrast, amongst primiparous animals, exposure durations of two hours appeared to result in the greatest numbers of astrocytes, with 24 hour and non-exposed animals showing roughly similar numbers. The effects of pup exposure at other times appeared to be much more inconsistent. In some cases, for example animals sacrificed on day five, longer term pup exposure seemed to produce substantial decreases in astrocyte numbers in the MPOA regions.

While, for the most part, the results of the present study are consistent with what is known about maternal behavior, there are some aspects which appear somewhat
puzzling. Specifically, three immediate questions arise from these results. The first of these has to do with the question of why postpartum pup exposure did not produce more substantial effects on astrocyte number. Behavioral studies have suggested that the amount of pup exposure needed to produce long-term changes in learning are very brief indeed (~1/2 hour). If astrocytic changes were an important component of the brain changes underlying maternal learning, we would expect that brief durations of pup exposure should be sufficient to produce substantial astrocytic changes.

The failure to find any significant main effect for exposure could be due to a number of reasons. First, it is possible that the hormonal changes associated with parturition overshadow, or mask, any effect of short term (2 to 24 hour) pup exposure on astrocyte number. As discussed earlier, many hormones, including estrogen, have a mitogenic effect on astrocytes, causing significant astrocyte proliferation. Thus, astrocytes likely undergo an increase around the time of parturition, due to increases in levels of estrogens. Such increases might make it difficult to discover any exposure based increase in astrocytes. It is interesting to note that, in the ten day group, some effects of pup exposure were seen, in the form of a significant interaction between parity and exposure. By ten days, postpartum hormone levels have declined to non-partum levels, perhaps removing the hormonal ‘mask’ and allowing effects of pup exposure to be seen.

Similarly, it is possible that hormonal changes produce substantial brain changes in postpartum animals, such that any animal under such conditions would show dramatic alterations in brain structure/functioning. What pup exposure might do is to make these alterations behaviorally relevant to the animal, such that, as a result of pup
exposures, animals are able to 'take advantage' of such changes. That is, behavioral changes may solidify hormonally induced brain changes (which occur in any animal primed by hormonal changes), such that animals given pup exposure are able to maintain these changes for a long period of time, while animals lacking this experience simply lose these brain changes following hormonal primes. Behavior occurring during postpartum hormone primes may also be able to 'take over' the function of brain changes resulting from such priming, with the end result being that a previously neutral change becomes able to control the occurrence of behavior.

To illustrate these points, an example is perhaps useful. It has been reliably shown that hormone changes, specifically increased levels of estrogen, produce substantial changes in hippocampal neurons, including changes in the number of dendritic spines and dendritic branching (Gould, et al, 1990; Woolley, et al, 1990; Woolley, et al, 1992; Woolley, et al, 1993). Normally, following the decline of estrogen, these changes decline to a baseline level. Such a phenomenon is quite similar to the types of synaptic changes which occur during development (namely the overproduction of synapses). Like what occurs during development, where environmental input determines which of these synaptic changes will remain, behavior which occurs during estrogen induced synaptogenesis may act to determine which of these synapses will survive. Maternal behavior would seem to have an unusually good ability to do this, since presumably, hormonal primes will cause maternal behavior to occur precisely at a time when such synaptic changes are also present.

A third explanation for the lack of a main exposure effect might have to do with the fact that the levels of exposure used in the present study were inadequate to
produce substantial astrocytic changes. In neither this study or our earlier study did animals given varying amounts of exposure (none, 2 hrs or 24 hrs in this study, or 24 hrs versus none in the earlier study) at the time of testing show significant differences in GFAP positive cells. Rather, differences appear to involve learning (in multiparous animals) which has taken place during the first parturition. One important difference between the primiparous and multiparous animals used in this study is the fact that, following their first parturition, animals were left with pups for a five day period before remating (a standard mating procedure for animals in our lab). Possibly, the parity effect observed in this study would not be found with animals given pups for a shorter period of time. At present, we are conducting a study similar to the present study involving longer term exposures (5 days) to help clarify this issue.

A final reason could involve the fact that, depending on what criteria is used to assess maternal behavior, the animals were not fully maternal during exposure. If one assumes that nursing behavior (crouching, lactating posture, etc.) is a significant component to maternal behavior, then many of the animals used in this study, who did not show extremely high levels of nursing behavior, would not be considered maternal. Whether or not the animals were maternal during the initial exposure is of great importance for maternal learning. If animals were not maternal, then, possibly, little learning took place during the initial exposures, and, hence, brain changes should not be too great. However, the animals did show fairly high levels of other component behaviors, and only seem lacking in terms of nursing behaviors. Further, it is not presently known whether the behavioral expression of nursing behavior is necessary for the occurrence of maternal learning. Rather, the main thing that appears to change in
experienced animals is the particular way that pups are viewed by the dam, and is demonstrated by decreases in the amount of time needed for such animals to display maternal behaviors. Nursing, because it tends to occur later in the interaction between a mother and her pups, after the animal has approached the pups, retrieved them, built a nest, etc., would not seem to be a crucial aspect of this learning.

The second question arising from these results is the question of why we failed to replicate the parietal effects found in our earlier study. In fact, parietal astrocyte counts were marginally significant (.1 < p > .05) in favor of primiparous animals, the opposite of what was found in the earlier study. To some extent this might be due to differences in the time at which animals were tested in each respective experiment. While both this study and our previous one are quite similar, our earlier experiment looked only at animals sacrificed at day five. In this study, the major parietal differences were seen at day one (which was not examined in our earlier study). At day five, no parity differences were seen between primiparous and multiparous groups, and, in fact, numbers were near identical in both groups.

Given that it appears that astrocytes change in response to maternal experience, as demonstrated in this experiment, and our earlier study, the most obvious question which follows is the question of how astrocytes contribute to maternal learning. There are many functions which astrocytes could be performing within the context of maternal behavior and learning. One interesting possibility could involve neurotrophic factors. Astrocytes are known to produce and secrete a number of neurotrophic factors, including nerve growth factor. While much has been learned about the role of neurotropic factors in learning associated with development and brain recovery, very
little attention has been placed upon the role that this substance might play in learning in normal adult animals. Some evidence of a facilitatory role for NGF on learning has been found in studies involving conditioned taste avoidance (Lipinski, et al, 1995). A role for NGF in maternal behavior, and possibly maternal learning, seems promising, considering the recent finding that lactating female rats show elevated serum levels of NGF (Alleva, et al, 1996). Given the ability of NGF to induce substantial changes in neural function and morphology, further studies in this area are likely to be of considerable use for the understanding of the underlying neural and pharmacological changes involved in maternal behavior.

There are, essentially, two ways that astrocytes could play a role in maternal learning; either indirectly or directly. Indirectly, astrocytes could be essential for setting, through such mechanisms as pH regulation, ionic regulation or the secretion of substances, the general substrate upon which brain functioning occurs. For example, through pH and ionic regulation, astrocytes could set well defined limits on the degree of neural activity which will be allowed to occur during any learning experience. In response to environmental input, astrocytes could depolarize and, therefore, produce extracellular pH changes which then regulate neural activity. In this manner, astrocytes could play an important role in such phenomena as state dependent learning, motivation and attention, all of which are likely quite important for maternal learning.

Directly, astrocytes could process information on their own, or in concert with neurons. Such information processing could be either short term or long term. In the short term, astrocytes may act as a first response system, wherein they produce quick responses to environmental input, for a period long enough to sustain responding until
the appropriate neural circuitry develops. Alternatively, it is possible that astrocytes could act to produce and maintain long lasting responses, and could, therefore, be responsible for some of the long term learning which takes place in the brain. Although, to date, there is no evidence which either supports or disputes this information processing role for astrocytes, astrocytes do seem to possess a number of features which would allow them to carry out an information processing role. In fact, given that astrocytes are able to migrate about the brain, and have an ability to proliferate throughout their life span, astrocytes would seem ideally suited for producing brain plasticity. Perhaps, as an animal ages, and as neural circuits solidify, astrocytic plasticity may become an increasingly important contributor towards learning induced plasticity.
References


Fleming, A.S., Gavarth, K., & Sarker, J. (1992). Effects of transections to the vomeronasal nerves or to the main olfactory bulbs on the initiation and long-term retention of maternal behavior in primiparous rats. *Behavioral and Neural Biology*, 57, 177-188.


preoptic area and ventral bed nucleus of the stria terminalis of postpartum rats. *Behavioral Neuroscience*, 109, 135-149.


Figure Legends

Figure 1. Schematic depicting experimental design and group sizes. Three variables, parity (primiparous versus multiparous), pup exposure (non-exposed, 2 hour exposed and 24 hour exposed), and day after exposure/manipulation that animals were sacrificed (one day - daysac1, five days -daysac5, and ten days - daysac10) were examined. All variables were completely crossed.

Figure 2. Schematic showing location of brain sites examined at the - .26 level. MPOA = medial preoptic level; AMPO = anterior medial preoptic area; AVPO = anterior ventral preoptic area; MPA = medial preoptic area; PAR = parietal cortex; VBNST = ventral bed nucleus of the stria terminalis.

Figure 3. Schematic showing location of brain sites examined at the 2.8 level. ACO = anterior cortical amygdaloid nuclei; PICO = posterior cortical amygdaloid nuclei; BLA = Basolateral amygdala; MEA = medial amygdala; CA1 = dorsal hippocampus; Hbl = lateral habenula; Mhb = medial habenula.

Figure 4. Graphs depicting mean numbers of astrocytes in MPOA area at each daysac condition.

Figure 4.a. (MPOA total) A significant main effect was found for parity in the MPOA (p < .012) at daysac 5, with multiparous animals showing higher numbers of astrocytes than primiparous animals. A significant two way interaction between exposure and parity was found at daysac10 (p < .011).

Figure 4. b (AMPO). A significant main effect in favor of multiparous animals was found for parity in the AMPO (p < .014) at daysac 5.

Figure 4. c (AVPO). A significant interaction between parity and pup exposure was found at daysac 10 (p < .005).

Figure 4. d (MPA). Significant differences between parity conditions were found in the AVPO at daysac five (p < .023). Additionally, an interaction between daysac and pup exposure was found at day ten (p < .029).

Figure 5. Graphs depicting mean numbers of astrocytes in the MEA. A significant main effect for parity was found in the MEA at day one (p < .03) and day ten (p < .008), with primiparous animals showing higher numbers of astrocytes than did multiparous animals. No other effects were found in this area.

Figure 6. Graphs depicting astrocyte numbers in the Lhb and Mhb. A main effect for parity was found in the Lhb (p < .017) and Mhb (p < .016) at day one.

Figure 7. Graphs depicting mean numbers of astrocytes in the parietal cortices A
significant main effect for parity was found in Par1 (p<.016) at day one with primiparous animals showing higher numbers of astrocytes than did multiparous animals. A similar parity effect was found in Par2 (p<.02) at day ten. No other effects were found in these areas.

Figure 8. Graphs depicting astrocyte numbers in remaining brain areas. No significant differences were found in these areas for any of the variables of interest.

Figure 8. a: BLA
Figure 8. b. ACO and PLCO
Figure 8. c. CA1
Figure 8. d. VBNST

Figure 9. Computerized image showing differences in GFAP immunoreactivity between primiparous and multiparous animals in the MPOA region. Brains were selected which most closely represented the mean number of astrocytes for the respective group from which it came. Brains are shown at a 10 x 15 magnification.

Figure 10. Computerized image showing differences in GFAP immunoreactivity between primiparous and multiparous animals in the MEA region. Brains were selected which most closely represented the mean number of astrocytes for the respective group from which they came.

Figure 11. Computerized image showing differences in GFAP immunoreactivity between primiparous and multiparous animals in the Lhb and Mhb regions. Brains were selected which most closely represented the mean number of astrocytes for the respective group from which they came.

Figure 12. Schematic depicting number of GFAP stained cells within the MPOA and Parietal cortices examined. Each dot equals ten astrocytes.

Figure 13. Schematic depicting number of GFAP stained cells within the amygdaloid nuclei. Each dot represents ten astrocytes.
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MPOA
The diagrams illustrate the number of cells showing GFAP for different exposure conditions and experimental days. The y-axis represents the number of cells (Mean + SE) showing GFAP, and the x-axis shows the exposure conditions, with bars indicating the mean and standard error for each group. The groups are labeled as 24 hr exposed, 72 hr exposed, and non-exposed.
Number of Cells (Mean + SE) Showing GFAP

Number of Cells (Mean + SE) Showing GFAP

Number of Cells (Mean + SE) Showing GFAP

MPA

Day 10

MPA

Day 5

MPA

Day 1

Prmip

Multiple

24hr exposed

non-exposed

24hr exposed

non-exposed

24hr exposed

non-exposed
Number of Cells (Mean + SE) Showing GFAP

Day 1

PAR1

Primip
Multip

PAR2

Primip
Multip

Day 5

PAR1

Primip
Multip

PAR2

Primip
Multip

Day 10

PAR1

Primip
Multip

PAR2

Primip
Multip

Legend:

- ■: 2hr exposed
- □: 24hr exposed
- ☐: non-exposed
Day 1

Day 5

Day 10

VBNST

VBNST

VBNST
AMYGDALA

Primiparous

Multiparous