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Injection of Neuroinflammatory Chemokine Alters Developmental Course of Social and Exploratory Behaviors of

Neonatal Rats

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Abstract

While the etiology of autism is unknown, the ten-fold increase in its incidence since the 1970s suggests novel

environmental factors. Recent human brain tissue analyses comparing patients with autism and neurotypical controls indicated the possible etiological role of chemokines, a large family of small chemoattractant cytokines that are involved in inflammatory processes and brain development. Using an animal model, the current study examined the effect of neonatal injection of one such chemokine, MIP-1 $\alpha$ , on the social and exploratory behavior of rats. The tests for social preference and exploratory frequency used in this study have numerous features, including shared neuroanatomical substrates (i.e., cerebellum and frontal cortex) with autism, that suggest their usefulness as models for autism in humans. For both the social and exploratory data, statistical analyses failed to reveal either a main effect of injection group or significant difference between the groups on any one test date. A profile analysis of the social preference data did, however, reveal a significant difference in group profiles over time. These different profiles evidence a link between increased immunoprotein levels in postnatal development and alterations in the developmental course of complex, higher order behaviors, as seen in autism spectrum disorders (ASD). These developmental differences help to elucidate the potential role of neonatal inflammation from maternal or childhood infections and vaccine allergies in the incidence of ASD.

Keywords: MIP-1 $\alpha$ ; social behavior; exploration behavior; rat; autism

## **Introduction**

Defined as a life-long developmental disorder, autism affects 3 to 5 in 1000 persons and is usually identified in early childhood between 18 and 30 months of age (Piven, 1997; Rapin, 1997; 2002; Trevarthen, 2000; Yeargin-Allsopp et al., 2003). Since there are no consistent anatomical markers to use for a diagnosis of autism, behavioral characterization is currently the only way to define the disorder. The widespread and diverse behavioral

characteristics of autism make it best classified as a spectrum disorder as no two individuals present identical symptoms (Rapin, 2002). Most commonly, autism is characterized by deficits in social interaction and communication, as well as obsessional mannerisms, behavioral inflexibility (Ijichi and Ijichi, 2004), and a higher incidence rate of epileptic seizures and increased risk of epilepsy throughout childhood (Besag, 2004; Rapin, 1997). Indeed, autism is often suspected after parents or pediatricians note an absence or delay of speech development, or a lack of normal interest in others.

Investigations into the neuroanatomical alterations in patients with autism have revealed several differences in areas such as the cerebellum (Bauman and Kemper, 1994), inferior olive nucleus (Bailey et al., 1998; Lee et al., 2002), and frontal cortex (Carper and Courchesne, 2000; Courchesne, 1997; Townsend et al., 2001). In addition, alterations of the acetylcholine system in the forebrain and cerebellum have also been revealed (Brambilla et al., 2003; Piven, 1997). Linking these anatomical differences to behavioral deficits, animal model studies performed on monkeys (Bachevalier, 1996), rodents (Bobee et al., 2000; File et al., 1993; Gerrits et al., 2000; Parikh et al., 2003; Pletnikov et al., 1999; Walker and Diefenbach, 2002), and sheep (Ferreira et al., 2001; Ferreira et al., 2003) have demonstrated that developmental lesions to the amygdala, basal forebrain cholinergic system, or cerebellum alter normal behaviors to that which is seen in patients with autism (see Gerlai and Gerlai (2003) and Murcia et al. (2004) for review of animal models).

Despite the neuroanatomical findings above, there is little consensus as to the main anatomical deficit found in autism, and a concrete cause of the disorder remains elusive. As such, much attention has been given to the role of genetics based on strong evidence from twin and family studies (Deuel, 2002; Korvatska et al., 2002; Lamb et al., 2000). While the high male-to-female ratio (3-5:1) of the autistic population (Deuel, 2002; Piven and Folstein, 1997; Volkmar and Pauls, 2003) and abnormalities on chromosomes 2, 7 and 15 (Gerlai and Gerlai, 2003; Lamb et al., 2000; Murcia et al., 2004; Turner et al., 2000; Volkmar and Pauls, 2003) point to a clear genetic influence in etiology of autism, specific genetic mechanisms have not been identified. This is specifically illustrated by the less than 100%

(~60-65%) concordance rate in monozygotic twin pairs (Gerlai and Gerlai, 2003; Piven and Folstein, 1997; Turner et al., 2000). In addition, “epistatic” genetic mechanisms alone cannot account for the ten-fold increase in the incidence rate of autism in the past 30 years (from 2-4 cases per 10,000 in the 1970s up to 3-5 per 1000 today) (Turner et al., 2000). The dramatic plasticity of the brain suggests the additional etiological role of environmental influences, especially when the successes of intensive behavioral treatment are considered (Howard et al., 2005).

One environmental influence that has received much attention is the group of immune system response mechanisms (e.g., as potentially induced by measles, mumps & rubella (MMR) vaccination; Deuel, 2002). Recent studies have shown that the immune systems of patients with autism are more active than those of their non-autistic counterparts, suggesting that they are more likely to produce detrimental immune responses when activated (Jyonouchi et al., 2001; Vargas et al., 2005). In a scenario postulated by Korvatska, et al. (2002), immune responses due to an environmental stimulus activate cytokines that cross the blood-brain barrier (BBB) to activate the local immune response of microglia, which can cause neuronal damage and alter behavior during development.

Chemokines (chemotactic cytokines) represent a class of cytokine that both initiates and participates in the immune response process and therefore present the ideal candidate for exploration of the aforementioned scenario, which has not been explored to date. Chemokines are a large family of small, secreted proteins (8-14kDa) that figure in the trafficking of leukocytes in physiological immunosurveillance as well as inflammation responses via inflammatory cell recruitment in different disease processes (Asensio and Campbell, 1999). They have also been heavily implicated in normal neurodevelopment, however, serving a role in regulating the number of surviving cells (Brenneman et al., 1999; Meucci et al., 1998) and orchestrating cellular migration. Coupled with their demonstrated influence in various other pathologies, such as multiple sclerosis and Alzheimer’s disease, such phenomena lend credence to the hypothesis that chemokines play significant and diverse functional roles (Ritter et al., 1995). In fact, a recent human brain tissue analysis demonstrated that various chemokines are upregulated in brains of autistic patients into adulthood, which suggests autism-like behaviors may be due to an ongoing immune response (Vargas et al.,

2005).

One chemokine in particular, known as macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), has been shown to be involved in cell proliferation, the survival of forebrain and cerebellar cells, and especially, CNS inflammation during immunochallenge (Asensio and Campbell, 1999; Brennehan et al., 1999) – bearing a direct relationship to the possible environmental etiology of autism. Therefore, in the current study we tested the hypothesis that focal injections of the chemokine MIP-1 $\alpha$  on either postnatal day (PND) 7 or 9 would alter the developmental course of social and exploratory behavior of rats when compared with saline-injected control rats. Injections were administered at PND 7 and 9, time-points that are critical periods during development when certain neuronal populations (cholinergic basal forebrain and cerebellum) are particularly sensitive to environmental insults and therefore may affect the development of behaviors relevant to autism.

## **Materials and methods**

### *Animals*

Pregnant inbred Sprague-Dawley rats (16-18 days gestation) were used in this study. Rat pups from five litters were raised with their dams until PND 31 in polypropylene cages (20 x 16 x 8.5”) with metal rung tops. Due to behavioral variability related to the estrous cycle (Bobee et al., 2000), only male rat pups were used for these experiments. The rats were housed under standard light conditions (12 hour light/12 hour dark with light on between 6 a.m. and 6 p.m.) and food and water were available ad libitum. All experimental protocols were in compliance with AALAC standards and approved by the Georgetown University Animal Care and Use Committee.

### *Surgery*

Rat pups from each litter were randomly divided into one of the following groups: MIP-1A injection on PND 7 (n=10), control injection on PND 7 (n=9), MIP-1 $\alpha$  injection on PND 9 (n=9), or control injection on PND 9 (n=7). Rat pups in the experimental condition for both PND 7 and 9 were injected with a total of 4 $\mu$ l of MIP-1 $\alpha$  into the lateral ventricles and pups in the control condition for both days were injected with a

total of 4 $\mu$ l of sterile saline (.9% NaCl) into the lateral ventricles. In order to administer the injections, rat pups were anesthetized with ice and placed in a Kopf stereotaxic apparatus. The incisor bar was placed 3.3mm below the interaural line. A midline sagittal incision was made in the scalp. Holes were drilled over the lateral ventricles bilaterally at bregma and +/-1.8mm lateral to bregma. A Hamilton syringe was then lowered 3.5mm ventral from top of brain. Rat pups were either injected with 2 $\mu$ l of MIP-1 $\alpha$  (0.4ng/ $\mu$ l in .9% NaCl) or 2 $\mu$ l of sterile saline into each ventricle over a two-minute period. After injections were completed, incisions were closed with Krazy glue and the rats were placed on a surgical heating pad until they warmed and regained movement. Pups were then reared with the dams until weaning at postnatal day 31.

### *Behavioral tests*

#### *Social preference test*

The social testing paradigm in this study, similar to those used by us and others (Bobee et al., 2000; Moy et al., 2004; Nadler et al., 2004; Parikh et al., 2003), was a social preference task with the aim of testing whether a rat prefers to visit its own territory or to explore the territory of an unknown rat when both territories are accessible during a 15-minute observation period. Rats were tested for social behavior two, four, six, and ten weeks after injections. The experimental apparatus (Habitest, Coulbourn Instruments) consisted of three chambers (each 20.5 cm x 10.5 cm x 12.2 cm): a home chamber [H], a social chamber [S] and an initial chamber [I]. The cages were arranged to allow an experimental rat in the middle, [I], box to walk freely between the [H] box and the [S] box (Figure 1).

Before testing began, a tray with bedding from the home cage of the test subject was placed in [H] and a social stimulus rat was placed in [S]. The social stimulus was completely novel to the test subject and a new social stimulus was selected for each subsequent presentation. A thick plastic partition, with six small holes (.25cm radius) and a larger hole (1.25cm radius) in the center, was placed one quarter of the way in both [H] and [S]. This partition allowed the test subject complete access to one quarter of [H] or [S] with minimal physical contact with the bedding or social stimulus. The entrance into each of chambers [H] and [S] from the initial chamber was surrounded by

photobeams which, when broken, recorded the number of entries and time spent in each of the cages by the test subject. All three compartments of the apparatus were thoroughly cleaned between each run with 70% ethanol and allowed to completely dry before the start of the next subject run. The total time spent in each chamber during the 15-minute test was recorded and analyzed by computer.

#### *Exploratory frequency test*

Following the paradigm of (Caston et al., 1998) and (Walker and Diefenbach, 2002) exploratory behavior was recorded in an experimental chamber (40cm X 40cm X 40cm; Figure 2). The experimental chamber contained a platform raised two centimeters above the bottom of the chamber that served as a floor plane on which animals were placed. The platform contained 16 holes, 1cm in diameter, arranged in a 4 X 4 array. Two rings of photocells were arranged around the perimeter of the chamber to record locomotion behavior along the plane of the floor of the chamber, as well as nose hole entries that extended below the chamber floor plane.

#### *Data analysis*

All comparisons regarding social preference were conducted between the [S] and [H] chambers only, without the inclusion of the time spent in [I]. The rationale for this was threefold. Firstly, since the rats were initially placed in [I], the time in this chamber would be artificially higher than the other chambers. Secondly, the rats had to pass through [I] in order to enter either [S] or [H], which would also artificially increase the time in this chamber. Finally, rats had the opportunity to stop in [I] without continuing on to either [S] or [H]. Therefore social preference was defined as the choice between the social and home territories only.

Because the social preference data was not normally distributed, a logarithmic transformation was applied to the data prior to analysis. So that effective comparisons could be made between groups, a measure of social preference was established, hereafter called 'sociability index.' The sociability index was derived according to the following formula:  $(S-H)/900$ , where S represents the time spent in the social chamber, H represents time spent in the home chamber, and 900 represents the total test time (900 seconds). This sociability index not only allows for an

assessment of social preference on the individual level by means of a difference score, but also for direct comparisons between animals as the difference score is represented as a percentage of total time. Based on these sociability index scores, the behavioral parameters were then analyzed using profile analysis and a two-way repeated measures analysis of variance (ANOVA).

Exploratory behavior was defined as the number of nose hole entries per minute of locomotion within the chamber during a 10-minute observation period (the number of holes explored and the total time the animal spent walking are both measured by the computer system (TruScan, Coulbourn Instruments)). This frequency measure allowed for the separation of spontaneous movement activity from the motivation of exploration, since rats with cerebellar vermis lesions are more hyperactive than controls (Caston et al., 1998; Walker and Diefenbach, 2002). Data from the exploration experiments were normally distributed (based on Kolmogorov-Smirnov tests) and were also analyzed according to profile analysis and a two-way repeated measures ANOVA.

## **Results**

A battery of health examinations was conducted following both MIP-1 $\alpha$  and saline injections to assess their effect on the pups and lactating dams. Dams showed no adverse responses towards the pups, accepting them back into the litter after injections and performing normal retrieval behaviors for pups briefly separated from the group within the home cage. These dams also raised the mixed litters (saline and MIP-1 $\alpha$  pups) normally. In addition, the MIP-1 $\alpha$  injected animals showed no developmental delays for body growth or the emergence of normal reflexive behavior, nor was there an increase in mortality of these pups.

### *Social preference*

A two-way repeated measures ANOVA revealed a statistically significant main effect for time ( $F(3,90) = 6.55; p < 0.01$ ). This main effect merely reflects the fact that normal development is, by nature, a dynamic process over time. As the focus of this study was the possible effect of the chemokine injections on different developmental days, no further post-hoc analyses were run on the within-subjects differences. The ANOVA did not reveal a statistically significant main effect for injection group ( $F(3,30) = 0.39; p > 0.05$ ) (Table 1). Furthermore, the

ANOVA failed to reveal a statistically significant interaction between time and group ( $F(9,90)=1.65$ ;  $p > 0.05$ ).

One-way ANOVAs comparing the sociability of the four groups at each time-point did not reveal any significant differences between the groups ( $p>0.05$  for all four test dates). Post-hoc  $t$ -tests, however, did reveal one significant difference between the PND7 experimental and PND9 experimental groups at week 2 ( $t(16)= -2.184$ ,  $p<0.05$ ). All groups displayed a social preference, as indicated by the positive sociability index scores, including the PND7 experimental group at week 2, in which the rats still spent an average of 75 seconds more time in [S] than in [H]. Despite the difference between PND7 and PND9 experimental groups at week2, there were no significant differences between control and experimental groups for either PND7 or 9 at any of the time-points ( $p>0.05$  for all control versus experimental comparisons).

Despite the lack of significant differences as tested by ANOVA, an analysis of the behavioral profile over time did, in fact, reveal a significant developmental difference in rat sociability ( $F(3,30)=4.62$ ;  $p < 0.01$ ). This difference shows an almost perfect inversion of profile for the PND 7 MIP-1 $\alpha$  group when compared with the profiles of the other three groups over time (Figure 3). This difference in profile reflects a statistically significant difference in change over time.

#### *Exploration behavior*

Similar to the results for social behavior, a two-way repeated measures ANOVA for exploration behavior revealed a statistically significant main effect for time ( $F(3,93)=17.61$ ;  $p < 0.01$ ). Again, no further post-hoc tests were conducted as the focus of this study was to investigate the effects of injection day and type. Also similar to the social behavior results, the ANOVA did not reveal a statistically significant main effect for injection group ( $F(3,31)=0.25$ ;  $p > 0.05$ ). Furthermore, the ANOVA failed to reveal a statistically significant interaction between time and group ( $F(9,93)=0.97$ ;  $p > 0.05$ ).

Unlike the analyses for social behavior, however, profile analysis for exploration behavior failed to show a significant difference in the profiles of the different groups ( $F(3,31)=2.71$ ;  $p > 0.05$ ). As seen in Figure 4, the

developmental profiles for exploration behavior are remarkably similar for each of the four groups.

## Discussion

Understanding the neurochemical changes that occur in a developing brain is essential for elucidating the emergence and course of childhood disorders like autism. As statistical analysis of the social behaviors profiles over time shows, the results of the current study demonstrate that injection of MIP-1 $\alpha$  on PND 7 had a significant effect on the developmental course of social behavior. Despite the fact that ANOVAs failed to show any significant differences between the groups, the use of profile analysis as a statistical model helped to elucidate the developmental changes in social behavior; this developmental perspective is particularly relevant in light of the fact that autism is fundamentally a developmental disorder. The fact that all four groups demonstrated an overall decrease in both sociability index and exploration frequency over time is most likely due to familiarization with the tests; Caston, et al. (1998) report similar familiarization effects with exploration behavior. Despite this overall decrease, the developmental trajectory of social behavior from week 2 to week 10 is not uniform across all four experimental groups, and the fact that the developmental course of the PND 7 experimental group is almost inverted is the most significant finding of the current study.

Furthermore, the day of chemokine injection had a clear effect on the developmental profile, as indicated by the significant differences in social behavior between PND7 and 9 experimental groups at week 2. As posited above, these two postnatal days represent developmental time-points when specific neuronal populations are particularly vulnerable to environmental insult, with cholinergic basal forebrain cells being most vulnerable on PND7 and cerebellar cells being most vulnerable on PND9. It seems, therefore, that injection of MIP-1 $\alpha$  on PND7 may have directly affected the development of cholinergic basal forebrain cells, in turn affecting the developmental trajectory of social behavior. By extension, it is plausible that the lack of any significant differences in the developmental profiles of exploration behavior is because the chemokine injections, either because of the type of chemokine used or the day injected, failed to affect neuronal populations that are important for exploration behavior. It remains unclear, however, whether the hypothesized differences in behavior would ultimately be due to anatomical

or neurochemical differences.

It must be recognized that this hypothesized developmental pathway is a hypothesis at best and it is critical that further research be conducted in an attempt to verify the differences in social behavior seen in the current study and to pinpoint their possible origins. Furthermore, these data are based on a single-dose injection of MIP-1 $\alpha$  and should be evaluated as such. The significance of these results emphasize the need for further exploration of this chemokine model of autism, and future research in this domain should include variations such as the administration of different concentrations of MIP-1 $\alpha$ , behavioral testing earlier in postnatal development, and repeated prenatal and postnatal administration of MIP-1 $\alpha$  to simulate repeated environmental exposure.

Despite the clear need for further research, the results of the current study are nonetheless interesting in light of our attempts to create accurate animal models, as autism is currently considered a developmental life-long condition of the human brain, with the possible onset of gene-environmental interactions beginning in late prenatal or early postnatal life (Courchesne et al., 2003; Ijichi and Ijichi, 2004). Based on several morphological and neurodevelopmental events, the development of the rat brain has been used to model the development of the early human brain. For example, the myelination, neuronal proliferation, and overall increase in brain size seen in humans between late gestation and 18 months postnatal occur in the second and third postnatal week in the rat (Vidair, 2004). Therefore, the neonatal rat has a brain roughly equivalent to a human brain midway through gestation, and a rat in the third postnatal week is similar, neurodevelopmentally, to a human at birth. Both human and rat brains have shown an increase in susceptibility to neurotoxins during early development (Carlezon et al., 2003; Vidair, 2004) when select neural populations in the cerebellum and hippocampus are undergoing neurogenesis and migration (Vidair, 2004).

As such, in the current study, increasing immunoproteins via intraventricular injections on either postnatal day 7 or 9 in the rat may represent an immunologic environmental challenge that may disrupt specific processes during certain sensitive or critical periods. Our results support this possibility by demonstrating that there is a difference in behavioral modification when MIP-1 $\alpha$  is injected intraventricularly just two days apart. This suggests that different brain mechanisms may be sensitive to this manipulation over these two days. Current studies are

underway to determine any anatomical changes, such as ectopic neuronal outgrowth (as seen in autism (Courchesne et al., 2003)) or increased glial infiltration that could occur in response to the neuroprotective (Asensio and Campbell, 1999; Brenneman et al., 1999) and proinflammatory (Asensio and Campbell, 1999) effects of MIP-1 $\alpha$ .

The ability of the immune system to function as an environmental challenge that alters normal neural development is a promising avenue of inquiry, since the function of the immune system has been implicated in the etiology of autism, with viral infections and vaccinations receiving most of the attention. Indeed, the possible link between the measles, mumps, rubella (MMR) immunizations and autism has been controversial for several years (Deuel, 2002) and has achieved recent prominence in the popular press. The possible response to viral infections in patients with autism is an intriguing question, since the immune systems of patients with autism have been shown to be more active, with a 30-70% increase in anti-brain autoantibodies in the CNS (Korvatska et al., 2002), as well as an increase in activated microglia within the cerebellum (Vargas et al., 2005). In addition, the immune systems of patients with autism produce more proinflammatory/counter-regulatory cytokines (such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 1 $\beta$  (IL-1 $\beta$ )) when their immune systems are stimulated with lipopolysaccharide (LPS) or microbial pathogens (Jyonouchi et al., 2001). Therefore, these results suggest that the immune system of autistic patients is more highly active and more likely to produce detrimental immune responses when activated.

Animal models of either direct or indirect maternal infection of developing rodents have corroborated existing human data by demonstrating profound anatomical and behavioral deficits following infection or in the offspring of infected dams (see Patterson (2002) for review). Preliminary results from another study in our lab indicate that maternally-administered bacterial infection via LPS injection produces alterations in the profiles of the offspring's social behavior that appear to be nearly identical to the contrasting profiles of social development seen in the current study. Further analysis of brain tissue via ELIZA will reveal whether or not the same receptors underlie the similar developmental effects of LPS and MIP-1 $\alpha$  insults. The similarity of their effects on social behavior, however, provides strong evidence for the fact that the behavioral deficits seen in developmental disorders, such as autism, may be due to the altered functioning of the immune system at specific points in late prenatal or early postnatal life.

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*Developmental Effects of MIP-1a or Saline Infusions on the Sociability Index*

Groups	2 weeks	4 weeks	6 weeks	10 weeks
PND 7 exp	0.08 (0.07)	0.18 (0.05)	0.24 (0.06)	0.07 (0.05)
PND 7 con	0.27 (0.12)	0.24 (0.03)	0.14 (0.04)	0.13 (0.02)
PND 9 exp	0.32 (0.05)	0.21 (0.04)	0.08 (0.06)	0.08 (0.04)
PND 9 con	0.27 (0.01)	0.14 (0.05)	0.11 (0.04)	0.11 (0.04)

Note: The numbers in parentheses indicate the standard error of the mean (S.E.M.)

Two-way repeated measures ANOVA	Within-subjects effects*: $F(3,90)=6.55, p < 0.01$  Between-subjects effects: $F(3,30)$ $=0.39, p > 0.05$
Profile analysis	$F(3,30)=4.63, p < 0.01$ Level 3 vs. Previous**: $F(3,30)$ $=4.06, p < 0.05$

\* indicates results that are significant at  $p < 0.01$

\*\* indicates results that are significant at  $p < 0.05$

Table 2

*Developmental Effects of MIP-1a or Saline Infusions on Exploratory Frequency*

<b>Groups</b>	<b>2 weeks</b>	<b>4 weeks</b>	<b>6 weeks</b>	<b>10 weeks</b>
PND 7 exp	8.36 (0.96)	8.53 (1.80)	6.48 (1.74)	3.43 (0.91)
PND 7 con	6.87 (1.12)	7.53 (0.61)	5.88 (0.82)	4.07 (0.77)
PND 9 exp	5.35 (1.41)	9.42 (1.53)	6.82 (0.71)	2.88 (0.57)
PND 9 con	7.24 (0.42)	8.88 (1.55)	6.28 (0.44)	4.00 (1.15)

Note: The numbers in parentheses indicate the standard error of the mean (S.E.M.)

Two-way repeated measures ANOVA	Within-subjects effects*: $F(3,93) = 17.61, p < 0.01$ Between-subjects effects: $F(3,31) = 0.25, p > 0.05$
Profile analysis	$F(3,31) = 2.71, p > 0.05$

\* indicates results that are significant at  $p < 0.01$

**Figure 1:** Social behavior experimental apparatus. Control and MIP-1 $\alpha$  pups are placed into the center compartment, [I], and a novel stimulus rat is placed in the social compartment, [S]. Home cage bedding from the test rat is placed in the home compartment, [H]. Photocells record both the number of entries into and the time spent in each compartment.

**Figure 2:** Exploration behavior apparatus. Rats are allowed to walk freely within the chamber for a 10-minute observation period. Photocells around the perimeter record animal movements around the chamber, as well as the number of nose hole entries.

**Figure 3:** Profile analysis revealed a significant difference in the profiles of the four different groups [ $F(3,30)=4.63, p<0.01$ ]. Visual analysis confirms this result, as the profile of the PND 7 exp group appears to be a near inversion of the profiles of the other three groups.

**Figure 4:** Profile analysis revealed no significant difference in the profiles of the four different groups [ $F(3,31)=2.71, p > 0.05$ ]. Visual analysis confirms this result, showing very similar developmental profiles for the four different groups.

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