Chapter 3

The Sensitive Period for Imprinting

3.1 Introduction

This chapter reports an experiment which investigated the ending of the sensitive period for visual imprinting in dark-reared chicks. This experiment was undertaken because, in studying the extension of the sensitive period, it was first necessary to determine an age when imprinting would no longer occur when using the particular imprinting paradigm adopted in this thesis.

The ending of the sensitive period for imprinting signifies a stage in the development of the chick when it will no longer respond to novel stimuli in a filial manner. The occurrence of the sensitive period for imprinting is not fixed to a specific age of the animal nor is it restricted to a specific time, rather it is dependent on many factors (see page 7). In visual imprinting, one of the most important factors in determining the length of the sensitive period is the previous visual experience of the animal. For example, Guiton (1959) showed that prior experience with broodmates adversely affects imprinting. In Guiton's experiment group-reared chicks did not imprint as well as isolated chicks, which suggests that they may have formed social preferences for their broodmates. A number of other studies have reported similar results showing that prior experience with broodmates interferes with imprinting (for example, Sluckin and Salzen, 1961; Gottlieb and Klopfer, 1962 and Polt and Hess, 1964). It is not only siblings or even moving objects for which chicks form preferences. Bateson (1964b) has shown that chicks will preferentially approach boxes that are patterned similarly to the walls of their home-cage compared to balls that are covered with an unfamiliar pattern. In the following experiment chicks of three different ages were tested for their ability to

imprint. This was done chiefly to determine an age when imprinting could no longer occur so that future experiments seeking to extend the sensitive period could use this age as a reference, provided that a similar experimental paradigm is followed. Only visual aspects of imprinting were examined and so, in order to prevent visual imprinting from occurring until exposure to the imprinting stimulus, all chicks were reared in the dark.

3.2 Methods

A total of 63 white leghorn × australorp chicks of both sexes were trained and tested in this experiment. These were derived from eggs, incubated in a forced draught, automatic turning incubator. Incubation and rearing conditions are detailed in Chapter 2 (see page 42). Different groups of chicks were imprinted on days 2, 4 or 6 post-hatching (day 2 is defined as the day following hatching). The number of chicks in each group is presented in Table 3-1. These chicks were drawn at random from five separate hatches. The imprinting procedure used is detailed in Chapter 2 (see page 46). In this experiment chicks were trained on either a stuffed hen or a box. Numbers tested in each group are set out in Table 3.1. Each group was tested for an imprinting preference twice. The first test occurred 1 h after training and the second test on the following day, 24 ± 2 hours later.

3.3 Results

3.3.1 Activity in training

The mean ± SEM activity of the chicks during the training period is presented in Table 3-1. The data was log transformed and analysed using a three factor analysis of variance with a repeated measure on the factor direction. The factors were the age of the chicks, the direction of movement and the stimulus used to imprint them. There was a significant main effect of age ($F_{2,57} = 4.41$, $p = 0.02$) and direction ($F_{1,57} = 30.79$, $p = 0.0001$), but no main effect of stimulus ($F_{1,57} = 0.057$, $p = 0.45$). There was a significant

interaction between the factors direction and age ($F_{1,57} = 3.96$, $p = 0.03$). There were no other significant interactions between any of the factors.

Table 3-1. Activity of chicks during training

Age	Stimulus	Number trained	Total activity (revolutions)	Activity towards (revolutions)	Activity away (revolutions)
Day 2	box	11	296 ± 71	245 ± 66	51 ± 10
	hen	10	664 ± 280	440 ± 158	224 ± 165
Day 4	box	10	553 ± 244	372 ± 215	180 ± 134
	hen	12	438 ± 176	386 ± 165	53 ± 49
Day 6	box	11	794 ± 143	658 ± 158	135 ± 14
	hen	9	622 ± 223	517 ± 232	134 ± 25

Table presents the mean ± SEM activity of the groups trained on either the box or the hen on one of days 2, 4 or 6 post-hatching. Also presented is the number of chicks trained in each group.

Fisher's LSD tests revealed that the total activity of the chicks imprinted on day 6 was significantly greater than the activity of the group trained on day 4 ($p < 0.05$). There were no other significant differences in the total activity of the groups, but there were significant differences between the groups when the direction of movement was taken into account. The group that approached the imprinting stimulus the most during training was the group trained on day 6. Their approach score was significantly greater than the approach of the group that was trained on day 2 ($p < 0.02$) and the group that was trained on day 4 ($p < 0.0005$). The approach towards the imprinting stimulus of the group trained on day 2 was also significantly greater than the approach score of the group trained on day 4 ($p < 0.01$). Furthermore, the chicks trained on days 2 and 6 both approached the imprinting stimulus significantly more than they moved away from it (day 2, $p < 0.0005$; day 6, $p < 0.01$), while there was no significant difference in the direction of activity of the chicks trained on day 4 ($p > 0.4$).

3.3.2 Activity in the preference tests

The total activity of the chicks in each of the preference tests is presented in Table 3-2, along with the percentage of chicks that reached the activity criterion in each test. The activity from each test was log transformed and analysed using a two-factor analysis of variance.

3.3.2.1 Test 1 hour after training

In the test 1 h after training there were no significant main effects due to the age at which the chicks were trained ($F_{2,33} = 1.76$, $p = 0.19$) or stimulus ($F_{1,33} = 1.85$, $p = 0.18$), and there was no significant interaction between these factors ($F_{2,33} = 1.06$, $p = 0.36$).

3.3.2.2 Test 24 hours after training

In the test 24 h after training there were no significant main effects due to the age at which the chicks were trained ($F_{2,41} = 1.44$, $p = 0.25$) or stimulus ($F_{1,41} = 0.54$, $p = 0.47$). The interaction between these factors was not quite significant ($F_{2,41} = 3.01$, $p = 0.06$).

Table 3-2. Activity of the chicks during the testing periods

Age	Stimulus	Test 1 hour after training			Test 24 hours after training		
		Reached activity criterion		Total activity (revolutions)	Reached activity criterion		Total activity (revolutions)
		n	%		n	%	
Day 2	box	10	91	11.8 ± 5	9	82	13.0 ± 8
	hen	7	70	13.8 ± 7	8	80	8.5 ± 8
Day 4	box	5	50	9.5 ± 7	8	80	4.7 ± 4
	hen	3	25	4.9 ± 3	8	67	7.0 ± 3
Day 6	box	7	64	5.1 ± 2	10	91	4.8 ± 8
	hen	8	89	1.1 ± 1	6	67	2.1 ± 1

Table presents the mean ± SEM activity in the tests 1 h and 24 h after training.
Also presented is the number (n) and percentage of chicks that reached the activity criterion in each of the tests.

3.3.2.3 Number of chicks that reached the activity criterion

The proportion of chicks reaching the activity criterion is presented in Table 3-2. This data was analysed using the weighted least squares approach that is described on page 55. The main effect of age treated was highly significant ($X^2 = 49.03$, df = 2, $p < 0.001$). Collapsing the data across stimulus and time tested it can be seen that on average only 56% of the day 4 chicks reached the activity criterion, while 81% of the day 2 chicks and 78% of the day 6 chicks reached the activity criterion. There were no main effects of stimulus ($X^2 = 0.29$, df = 1, $p > 0.10$) or time tested ($X^2 = 0.10$, df = 1, $p > 0.10$). There were no significant interactions between any of the factors; age treated × stimulus ($X^2 = 3.99$, df = 2, $p > 0.10$), age treated × time tested ($X^2 = 0.15$, df = 2, $p > 0.10$), stimulus × time tested ($X^2 = 1.25$, df = 1, $p > 0.10$).

3.3.3 Percent preference scores

The mean ± SEM percent preference scores in both testing periods are presented in Figure 3-1. The percent preference scores from each test were analysed separately using a two-factor analysis of variance on the arcsine transformed data. The factors in the analysis of variance were age of the chicks at training (age) and stimulus used in training (stimulus).

3.3.3.1 Test 1 hour after training

In the test 1 h after training there was a significant main effect of age ($F_{2,33} = 6.17$, $p = 0.005$), but no significant main effect due to stimulus ($F_{1,33} = 0.31$, $p = 0.58$). There was no interaction between these factors ($F_{2,33} = 0.53$, $p = 0.59$). Fisher's LSD test showed that without respect to the stimulus used to train the chicks, those chicks trained on day 2 had significantly higher percent preference scores than the chicks trained on day 4 and day 6 ($p < 0.05$).

T-tests using the arcsine transformed data were used to determine which groups had percent preference scores that differed significantly from the no-preference level. Both the box-trained and the hen-trained groups that were trained on day 2 showed a

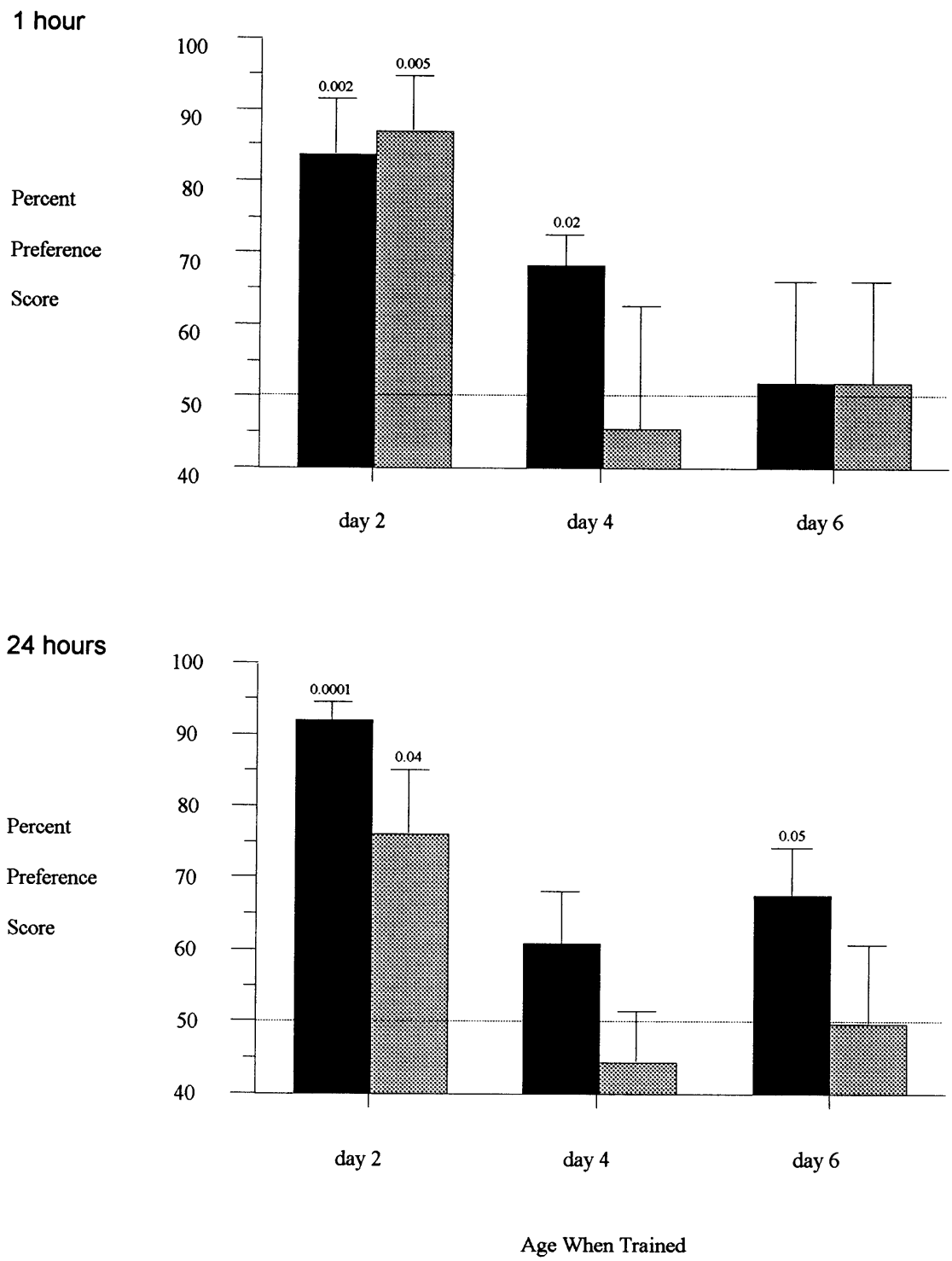


Figure 3-1. Mean ± SEM percent preference scores of the groups trained on days 2, 4 or 6 post-hatching. The chicks were trained on the box (black bars) or the hen (grey bars). In both of the testing periods the groups trained on day 2 showed significantly higher preferences for the imprinting stimulus than the groups trained on day 4 or day 6. The dashed line represents the no-preference level of 50%. The values above the error bars represent the p values of two-tailed t-tests between the group and the no-preference level.

3.4 Discussion

Using the imprinting paradigm which will be followed throughout this thesis, it was shown that the ability of untreated chicks to imprint declines between days 2 and 4 post-hatching. Those chicks trained on day 2 had significantly higher percent preference scores than the chicks trained on days 4 or 6 post-hatching in both the test 1 h and 24 h after training. Furthermore, in both testing periods the day 2 chicks trained on the box or the hen showed a significant preference for the imprinting stimulus compared to the no-preference level of 50%. In contrast, the only significant preference for the imprinting stimulus that was shown by chicks trained on day 4 post-hatching was that of the box-trained chicks in the test 1 h after training. This group showed no preference for the imprinting stimulus in the test 24 h after training. The only significant preference for an imprinting stimulus shown by chicks trained on day 6 post-hatching was that of the box-trained chicks in the test 24 h after training.

It is notable that of the chicks trained on day 4 or day 6 post-hatching it was only the box-trained chicks that showed a significant preference for the imprinting stimulus. Irrespective of age there was a consistent tendency for box-trained chicks to have higher imprinting scores than the chicks trained on the hen. In fact, there was a main effect of stimulus in the test 24 h after training which is attributed to the significantly higher percent preference scores of the box-trained chicks. Although the box proved to be a more effective imprinting stimulus than the hen, the group of chicks trained on day 2 post-hatching displayed a strong preference for the imprinting stimulus irrespective of what that stimulus was. In contrast, during both tests, the chicks trained on day 4 and day 6 showed significantly less preference for the stimulus on which they had been trained compared to the day 2 chicks. Thus, although the chicks were kept in darkness except when primed, trained and tested, their ability to imprint declined significantly after day 2 post-hatching.

These results concur well with those of others who have found that the sensitive period was restricted to the first few days after hatching (for example Moltz and Stettner,

1961; Fabricius and Boyd, 1954; James, 1960; Jaynes, 1957; Brown and Hamilton 1977; Boyd and Fabricius, 1965 and Ramsay and Hess, 1954). Thus, in this experiment there did not appear to be an appreciable extension of the sensitive period due to dark-rearing.

There have been reports of light deprivation extending the sensitive period to at least day 7 post-hatching (e.g. Sluckin, 1962; Case and Graves, 1978) but the studies are not directly comparable with the present study because different training and testing procedures were used. The training and testing procedures alone could influence the length of the sensitive period that was measured. For example, Case and Graves (1978) reported that isolated, light-reared chicks would follow a moving-clucking model up to day 4 post-hatching. Chicks dark-reared to day 4 post-hatching and thereafter light-reared in visual isolation, followed the moving-clucking model up to day 7. In the present experiment only a visual imprinting stimulus was used and the sensitive period for imprinting on this stimulus was shown to end sometime between days 2 and 4 post-hatching. Had an auditory imprinting stimulus been used as well, it is possible that imprinting could have occurred in older chicks. However, it is likely that an age would be reached when imprinting on a combined auditory and visual stimulus is no longer possible.

The important question is: how does the sensitive period for imprinting end? Perhaps the most widely held view is that it is the process of imprinting itself which ends the sensitive period (Bateson, 1990, Horn, 1990). This is true in most instances as chicks are reared in an environment where they are exposed to any number of stimuli on which they may imprint. However, in the present experiment the fact that chicks reared in complete darkness lost their ability to imprint sometime between days 2 and 4 post-hatching tells us that the sensitive period may end without visual imprinting occurring. Therefore, if the only way that the sensitive period for imprinting can end is through imprinting itself, then in the present study those groups that did not imprint could only have imprinted on the dark, or at least they may have become accustomed to their rearing environment (cf Bateson, 1964b).

Alternatively, it is possible that during the period of dark-rearing, auditory imprinting could have occurred. Boyd and Fabricius (1965) have shown that ducklings are responsive to auditory calls for as long as 10 days post-hatching. It is known that auditory imprinting enhances the response to a visual stimulus when the two stimuli are presented together. In some instances the auditory component of the paired audio-visual imprinting stimulus has been reported to be the more powerful component (Gottlieb, 1971; Case and Graves, 1978). However, recent evidence suggests that auditory stimuli may not play as important a role as was once thought. van Kampen and Bolhuis (1991) investigated auditory imprinting using a rigid experimental approach. The auditory stimulus used was a pure tone, either 350 Hz or 600Hz. In a choice test, discrimination between a familiar tone and an unfamiliar tone occurred only in those chicks that were trained on a tone and a visual stimulus in combination. Chicks that were trained on a tone only did not show a discriminating preference between the familiar tone and an unfamiliar one. Furthermore, a recent review by Bolhuis and van Kampen (1992) has evaluated a number of studies in which auditory imprinting has been involved in filial imprinting. Accepting only those studies that were more akin in design to modern imprinting studies, they concluded that the auditory component of filial imprinting is not as important as the visual component. Auditory stimulation undoubtedly has a role in filial imprinting although it may be restricted to increase the level of arousal of the chicks, thereby facilitating the imprinting process.

The chicks in the present study were dark-reared for a significantly longer period of time than the chicks in the experiment of van Kampen and Bolhuis (1991), which were dark-reared for 30 hours before imprinting. The stimulation of the auditory system during the period of dark-rearing, coupled with the lack of stimulation of the visual system, could have allowed the auditory system to exert an unusually high level of input for imprinting. The interaction and competition between the visual system and the auditory system in the bobwhite quail chick, shown by Lickliter (1990) and described in Chapter 1 (see page 8) provides a good example of what could have occurred to the chicks in the present study. When the visual system received additional stimulation, its

development was enhanced and the phase of development during which a combined auditory and visual maternal model was needed to elicit approach behaviour occurred earlier than normal. It stands to reason that by depriving chicks of visual stimulation the reverse may happen. The auditory system may continue to develop and, in effect, may limit the importance of the visual system in filial imprinting. It is possible that in the present experiment the stage in the development of the chick, during which visual imprinting could occur, declined without a visual attachment being formed.

This view is in accord with the view held by Moltz (1963). He stated that "the imprinting pattern is organised during ontogeny through the progressive interaction between the developing organism and its sensory environment" (Moltz, p. 125, 1963). A similar view held by Hess (1973) was that the sensitive period was determined by a genetically sequenced chain of events, the progression through which could be slowed down or speeded up by manipulating the sensory environment of the animal (Hess, pp. 385-386, 1973). Hess' earlier conception was that it was "...maturationally determined..." (Hess, p. 385, 1973); that is, it is a function of the age of the animal and not necessarily due to the animal's experience. Curiously Hess (1973) distanced himself from the position of Moltz (1963) who, in my opinion, had proposed much the same mechanism. In the 1963 paper Moltz actually addressed previous criticisms of his viewpoint by Hess (1958, 1959a) claiming that Hess had misconstrued the epigenetic view of imprinting in claiming that it minimised the involvement of endogenous events. Both Hess and Moltz held the view that sensory experience is involved in the temporal limits of the sensitive period. Hess (p. 386, 1973) suggested that there are progressive stages of development which provide an order of maturational events, although not narrowly confined to specific ages (as he had originally thought).

The recent work of Lickliter (1990) shows that in the bobwhite quail developmental phases can be influenced by the appropriate sensory experience. In the present experiment one might argue that by depriving chicks of visual experience, the phase during which they would normally have visually imprinted passed without imprinting occurring. This does not provide evidence for an endogenous ending of the sensitive

period because as has been previously mentioned, it is possible that auditory imprinting occurred during the period of dark-rearing and this could have contributed to the ending of the sensitive period for imprinting.

Another explanation for the ending of the sensitive period is the increase in fear behaviour of the animals. The percentage of animals reaching the activity criterion at each age was presented in Table 3-2 and it is notable that in the groups trained on day 4 post-hatching, fewer chicks reached the activity criterion in the test 1 h after training than in the test 24 h after training. The number of chicks responding in the test situation may be influenced by their level of fear. Fear and avoidance behaviour may not yet be expressed in day 2 chicks and this may contribute to their superior imprinting. It has been reported that avoidance behaviour increases up to day 4-6 post-hatching (Schaller and Emlen, 1962; Bateson 1964c), and the development of fear behaviour has been suggested as being a significant factor in the declining ability to imprint (Hess, 1959b). The procedure involved in testing the chicks may have been particularly fear inducing, as they were confronted with a stimulus in either direction. Chicks that were fearful during the testing periods may have been expected to either freeze or, if they were imprinted on one of the stimuli one might expect them to run towards the familiar stimulus. In the second test, the percentage of chicks that reached the activity criterion increased, which may indicate their familiarity with the testing environment and hence a reduction in fearfulness.

If the day 4 and day 6 chicks had increased levels of fear this should have been reflected in the activity of these chicks during the training period, with the expectation that the activity of the older chicks may have been suppressed. However, this was not so. Indeed, the opposite occurred. The group of chicks that were trained on day 6 actually had higher levels of activity directed towards the stimulus in training than the two groups of younger chicks. The lower activity of the day 2 chicks may simply be accounted for by their poorer locomotor ability, and not because they were inhibited from approaching the imprinting stimulus. In fact, in training the day 2 and day 6 chicks directed significantly more of their activity towards the imprinting stimulus than away

from it, showing that the stimulus elicited approach behaviour, not fear behaviour in these groups. This was not the case for the chicks trained on day 4, as their activity directed towards the imprinting stimulus during training was not significantly different from their activity directed away from the imprinting stimulus. This may indicate that the day 4 chicks had heightened levels of fear, compared to day 2 chicks, and this could have contributed to their lack of imprinting.

3.5 Conclusion

The ending of the sensitive period for filial imprinting was determined using the imprinting paradigm which was followed throughout this thesis. It was not possible to imprint chicks older than day 2 post-hatching using the imprinting procedure employed in this thesis. An experience dependent mechanism is indicated because, although chicks may not have visually imprinted in the traditional sense, they may have formed some preference for their dark-environment, or other modes of sensory stimulation may have acted to close the sensitive period.

Chapter 4

An Investigation into the N-methyl-D-aspartate Binding Changes in the Intermediate Medial Portion of the Hyperstriatum Ventrale Following Treatment with Ketamine-Xylazine and Exposure to an Imprinting Stimulus on Day 8

4.1 Introduction

The results presented in the pilot experiment indicated that the sensitive period for filial imprinting could be extended by treatment with a mixture of KX. Chicks treated with the mixture of KX during the first 2 days post-hatching were able to imprint on day 8 post-hatching, while control chicks were unable to imprint at this time. As an increase in NMDA receptor number has been shown to occur in the left IMHV of chicks that were imprinted on day 2 post-hatching (McCabe and Horn, 1988, 1991; Johnston *et al.*, 1993), it is reasonable to ask whether these changes also occur in chicks that are able to imprint on day 8 because of the prior treatment with KX. This may indicate whether similar subcellular mechanisms are involved in imprinting on day 2 and day 8.

The increase in NMDA-sensitive L-glutamate receptors in the left IMHV of day 2 chicks following exposure to an imprinting stimulus has only been shown to occur between 6 and 8½ h after training (McCabe and Horn; 1991). Prior to this period, no change in NMDA receptor binding was shown, and it is not yet known if the changes persist after 8½ h.

McCabe and Horn suggest that the increased binding in the IMHV is due to a greater number of NMDA receptors and not to a change in receptor affinity. However, Johnston *et al.* (1993) have recently shown that there is an increase in both the number of L-glutamate receptors and in the affinity of binding to L-glutamate receptors in the left

hyperstriatum ventrale of imprinted chicks. Whatever the mechanism, it is clear that the process of imprinting is associated with an increase in NMDA receptor binding in the left IMHV.

It is possible that the mixture of KX has exerted its effects through a modulation of the NMDA receptor early in life. Ketamine belongs to the family of phencyclidine (PCP)-like compounds that act as non-competitive antagonists of the NMDA receptor (Harrison and Simmonds, 1985; Martin and Lodge, 1985). Thus, the ketamine component of the mixture of KX has the potential to modulate NMDA receptor activity.

Figure 4-1 is a schematic representation of the NMDA receptor. Binding studies in the rat forebrain have shown that PCP-like compounds, such as ketamine, bind in a distribution identical to that of the NMDA receptor (Maragos *et al.*, 1988). The site of action of these compounds is thought to be within the Ca^{2+} channel associated with the NMDA receptor (Lodge *et al.*, 1989, Loo *et al.*, 1986).

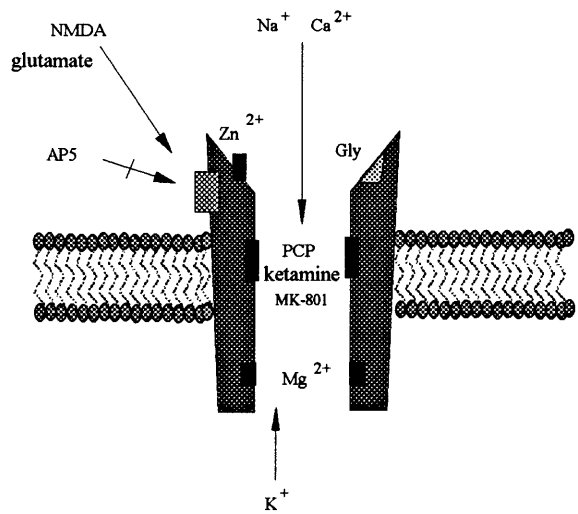


Figure 4-1. Schematic diagram of the NMDA receptor.
The cation channel is permeable to Ca^{2+} and Na^{+} it is blocked by Mg^{2+} in a voltage-dependent fashion. The PCP binding site is located within the channel and serves as a non-competitive antagonist, ketamine and MK-801 also act at this site. AP5 is a competitive antagonist for the NMDA binding site. Glycine and Zn^{2+} modulate the action of the receptor. Modified from Foster and Fagg (1987).

The most potent of the PCP-like non-competitive NMDA receptor antagonists is MK-801 (Wong *et al.*, 1986). In the experiment reported in this chapter, [^{3}H]-MK-801 was used to determine the density of NMDA receptors in the IMHV of chicks that had been exposed to an imprinting stimulus.

4.2 Methods

The biochemical aspects of this work were performed in the Laboratory of Dr. Peter Dodd, at the Royal Brisbane Hospital Foundation's Clinical Research Centre, Herston, Queensland. His assistance is gratefully acknowledged.

4.2.1 Subjects

A total of 79 chicks of either sex were used in this experiment. These came from eight separate hatches, and were randomly allocated to two treatment groups. At 10 ± 1 h post-hatching the chicks were treated intramuscularly with either the KX mixture (55 mg/kg ketamine and 6 mg/kg xylazine, made up to a final volume of 0.1 ml by the addition of sterile pyrogen free 0.9% saline) or 0.1 ml of the vehicle. A total of 23 KX-treated chicks and 26 saline-treated chicks were exposed to an imprinting stimulus on day 8 post-hatching. A further 14 KX-treated chicks and 16 saline-treated chicks were reared to day 8 and were killed without being exposed to any visual stimulation.

Rearing conditions were identical to those used throughout this thesis, and are described in full on page 43. Briefly, the chicks were hatched and reared in complete darkness. Ten to twelve hours after treatment they were transferred from the incubator to group rearing cages. Both the incubator and the rearing cages were situated inside the same room. Thus, throughout the rearing period chicks were totally deprived of light.

4.2.2 Imprinting

On day 8 post-hatching the chicks were removed from the dark-room in groups of four or five and transported in a light proof box to the adjoining imprinting room. Full

details of the imprinting methods may be found on page 46. Briefly, each chick was *primed* and then placed in an imprinting wheel for a period of 2 h, during which time they were exposed to either the rotating stuffed hen or the rotating red and black box (see page 51). After training, the chicks were returned to the dark-rearing room where they had access to food and water.

Testing, using a simultaneous choice test (see page 51) occurred 8 h after the end of training. It was decided to test at this time because, as mentioned in the introduction to this chapter, previous studies of NMDA receptor levels in the IMHV have demonstrated an increase in NMDA receptor number only between 6 and 8½ h after training (McCabe and Horn, 1991). Time periods after this have not been examined, and it is not known if the changes in NMDA receptor number would be present at a later time.

The preference of the chicks for the imprinting stimulus was determined using a simultaneous choice test (see page 51 for full methods) and was expressed as a percentage of the total activity of the chick in the test (as described on page 54). Percent preference scores and [^{3}H]-MK-801 binding were only analysed from those chicks that reached the activity criterion (see page 53 for an explanation of the activity criterion). Nineteen KX-treated and 22 saline-treated chicks reached the activity criterion.

4.2.3 Tissue collection

Immediately after a chick was tested it was killed by decapitation, and the left and right IMHV were rapidly dissected according to the method of Horn (1991 pp. 44-48). Only a brief description will be given here. The skin overlying the skull was cut along the midline and pulled back to reveal the skull. The skull of day 8 chicks is slightly ossified but can be easily cut with a pair of fine pointed scissors enabling the dorsal part of the skull to be gently lifted off exposing the brain. With a fine pair of microsurgery scissors a cut in the posterior wall of the ventricle was made and continued medially to the midline, then forward to approximately three-quarters up the length of the hemisphere. The medial wall of the ventricle can be pushed down between the hemispheres and the roof of the ventricle may be pushed laterally to expose the

ventricular surface of hyperstriatum ventrale. The next step is to make the incisions that defined the boundaries of the IMHV. These were made using a Swann-Morton No. 11 scalpel blade. The posterior cut was made at right angles to the midline, approximately 1 mm anterior to the posterior pole of the hemisphere. The second cut was in the same plane, but approximately 1 mm anterior to the mid point of the of anterior and posterior poles of the hemisphere. It was 3 mm deep and extended 2 mm laterally. The next cut was made in the sagittal plane approximately 0.5 mm from the midline with the scalpel blade overlapping the first and second cuts and angled slightly such that a wedge of tissue was removed, and this was mainly IMHV. Each IMHV was placed in a labelled specimen jar containing ice-cold 0.32 M sucrose and frozen at -18° C for 1 h, before storage at -70° C.

The amount of tissue needed for the binding procedure required that two IMHV's be combined to form one sample. In order to do this chicks within each group were given a rank according to their percent preference score and their total activity. They were paired primarily according to their percent preference scores, but where ties arose the activity of the chicks was used to decide which chicks should be paired. From these pairs of chicks a left IMHV sample and right IMHV sample were formed, each sample comprised two like-sided IMHV's. From the saline treated group that was exposed to the imprinting stimulus, 11 samples from each side were formed, and from the KX-treated group 9 samples from each side were formed. The non-exposed chicks were randomly paired into six saline treated and seven KX treated pairs.

4.2.4 [^{3}H]-MK-801 binding assay

Each sample was thawed at 37° C and homogenised in 1:40 w/v cold 0.32 M sucrose. The homogenate was centrifuged at 804 g for 10 minutes at 4° C. The resulting supernatant was centrifuged at 20 000 g for 20 minutes at 4° C. The pellet was resuspended in 40 vols of distilled water and frozen at -80° C for at least 30 minutes.

Frozen membrane preparations were thawed and centrifuged at 82 000 g for 30 mins at 4° C. The pellet was resuspended in 300 µl of 5 mM Tris-HCl, pH 7.4 (buffer) at

room temperature. [^{3}H]-MK-801 binding assays were performed in duplicate, at a thermostatically controlled room temperature of 23-24° C. The reactions occurred in U–shaped 96–well microtitre plates in a total volume of 250 µl. Wells measuring total binding contained 30 µM glycine, 10 µM glutamate, 2.5 nM [^{3}H]-MK–801 (New England products-Dupont, specific activity 25.7 Ci/mmole) in 200 µl buffer. Fifty µl of membrane protein was added to each well. Wells measuring non-specific binding contained in addition 500 nM unlabelled MK-801 to saturate the binding site.

The binding reaction was stopped after 45 minutes by filtration through Whatman GF/B filters, followed by cold buffer, using a Brandell 24-Cell Harvester. The filters were placed in scintillant (Packard Emulsifier Safe™) and radioactivity was measured on a Beckman β–scintillation counter with on-board quench correction.

The average non-specific binding per sample, measured in disintegrations per minute (dpm) was subtracted from the average total binding in each sample to obtain the specific binding. Specific binding was standardised against the amount of protein present in each well, determined using the method of Lowry *et al.* (1951).

One hundred µl of membrane protein suspension was set aside for the protein assay. This was centrifuged at 16 000 rpm for 10 minutes at room temperature and the resulting pellet was suspended in 1 ml of 3M NaOH and incubated at 70° C for one h. Every fifteen minutes the suspension was briefly vortexed. In duplicate, 100 µl of the suspension was incubated for 10 minutes with 2% Na_2Co_3, 0.01% $CuSO_4$ and 0.02% sodium tartrate in a volume of 2 ml. One hundred µl of Folin's Cacadolate reagent was added to the tubes which were vortexed immediately and incubated at room temperature for 45 minutes. A standard curve was obtained using bovine serum albumin in the assay. Absorbance was read at 750 nm.

4.2.5 Statistical methods

To determine the effect of treatment and stimulus on the likelihood of an animal reaching the activity criterion a logistic regression analysis was performed using the SPSS statistical package. The percent preference scores were arcsine transformed and

analysed using a two-factor analysis of variance, the factors being treatment and stimulus used in training. It was also of interest to determine if the individual means of each group varied significantly from the no-preference level of 50%, and this was examined using a t-test. A directional t-test for the KX-treated group, predicting that the mean would be significantly greater than the 50% or no-preference level, was used, while a non-directional test was used for the saline-treated group.

Following the procedure of McCabe and Horn (1991) separate analyses were performed on the binding data from the left and right IMHV. A square root transformation to correct for homogeneity of the data was indicated by the Box-Cox diagnostic (Box and Cox, 1964). The data was then analysed using a two-factor analysis of variance. Where significant variation was indicated, follow-up tests were performed using Fisher's LSD test. Paired t-tests using the transformed data were performed to compare the density of NMDA receptors in the left and right IMHV of each group.

4.3 Results

4.3.1 Activity in training

The activity of the chicks during the training period is presented in Table 4-1. The data was log transformed and analysed using a three-factor analysis of variance with a repeated measure on the dependent variable. The main effect of direction was significant ($F_{1,44}$ = 13.32, p = 0.001). Significantly more activity was directed towards the imprinting stimulus than away from it. There were no main effects of treatment ($F_{1,44}$ = 2.63, p = 0.11) or stimulus ($F_{1,44}$ = 0.19, p = 0.66) and no significant interactions between any of the factors.

4.3.2 Activity in the preference tests

The activity of the chicks in the preference test is presented in table 4-2. The data was log transformed and analysed using a two-factor analysis of variance. There was no

Table 4-1. Activity during the training period

Treatment	Stimulus	Number trained	Total activity in training (revolutions)
Saline	box	14	541 ± 150
	hen	12	565 ± 145
KX	box	10	413 ± 120
	hen	13	278 ± 56

Mean ± SEM total activity in the training period. Activity was measured in revolutions of the imprinting wheel. There was no significant difference in the activity of the chicks during the training period. The table also presents the number of chicks trained in each group.

significant main effect of treatment ($F_{1,35} = 0.03$, $p = 0.87$) or stimulus ($F_{1,35} = 0.78$, $p = 0.38$), nor was there an interaction between these factors ($F_{1,35} = 0.24$, $p = 0.62$).

Table 4-2. Activity in the preference test

Treatment	Stimulus	Reached activity criterion		Total activity (revolutions)
		n	%	
Saline	box	13	93	1.0 ± 0.3
	hen	7	58	9.3 ± 9.0
KX	box	6	60	1.5 ± 0.7
	hen	13	100	0.6 ± 0.1

Mean ± SEM activity measured in revolutions of the imprinting wheel in the preference test 8 h after training for saline and KX treated chicks trained on the box and the hen. Also presented is the number (n) and percentage of chicks that reached the activity criterion in each of the groups.

4.3.2.1..Number of chicks that reached the activity criterion

The proportion of chicks that reached the activity criterion is presented in Table 4-2. A logistic regression analysis revealed no significant main effects of treatment ($W = 0.0976$, d.f. = 1, $p = 0.75$) or stimulus ($W = 0.0967$, d.f. = 1, $p = 0.76$). There is no significant interaction between the factors treatment and stimulus ($W = 0.069$, d.f. = 1, $p = 0.79$). In looking at the data one might expect that there would be a significant interaction between treatment and stimulus. The statistical analysis indicates that this is

not the case, but one may speculate that had the sample sizes been larger a significant interaction may have resulted.

4.3.3 Percent preference scores

The percent preference scores of these chicks are presented in Figure 4-2. A two-factor analysis of variance was used to analyse the data. The factors were treatment and stimulus used in training. There were no significant main effects due to treatment ($F_{1,35}$ = 1.03, p = 0.32) or stimulus ($F_{1,35}$ = 0.15, p = 0.71), nor was there an interaction between these factors ($F_{1,35}$ = 0.24, p = 0.63). Thus, 8 h after training there was no significant difference between the groups, and no effect of the stimulus used to train the chicks.

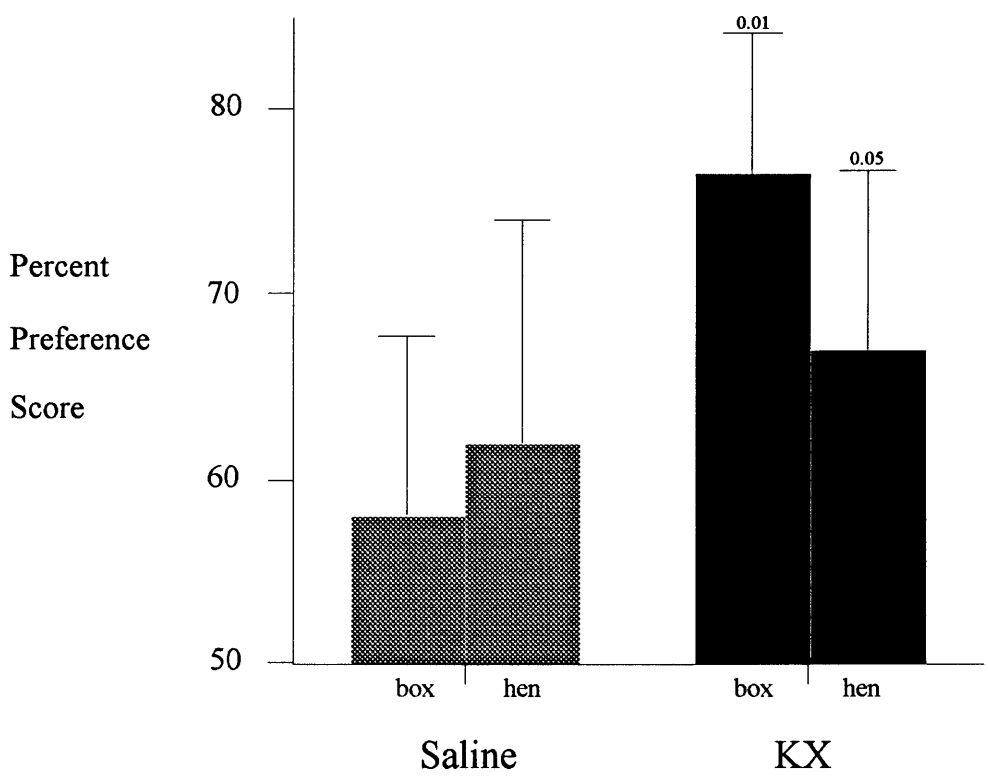


Figure 4-2. Mean ± SEM percent preference scores of the ketamine-xylazine-treated and the saline-treated groups from which IMHV samples were obtained.
The grey bars represent the saline-treated groups, the black bars represent the KX-treated groups. The values above the error bars represent the p-values of t-tests between the group scores and the no-preference level (one-tailed t-tests). Only the significant values are presented on the figure.

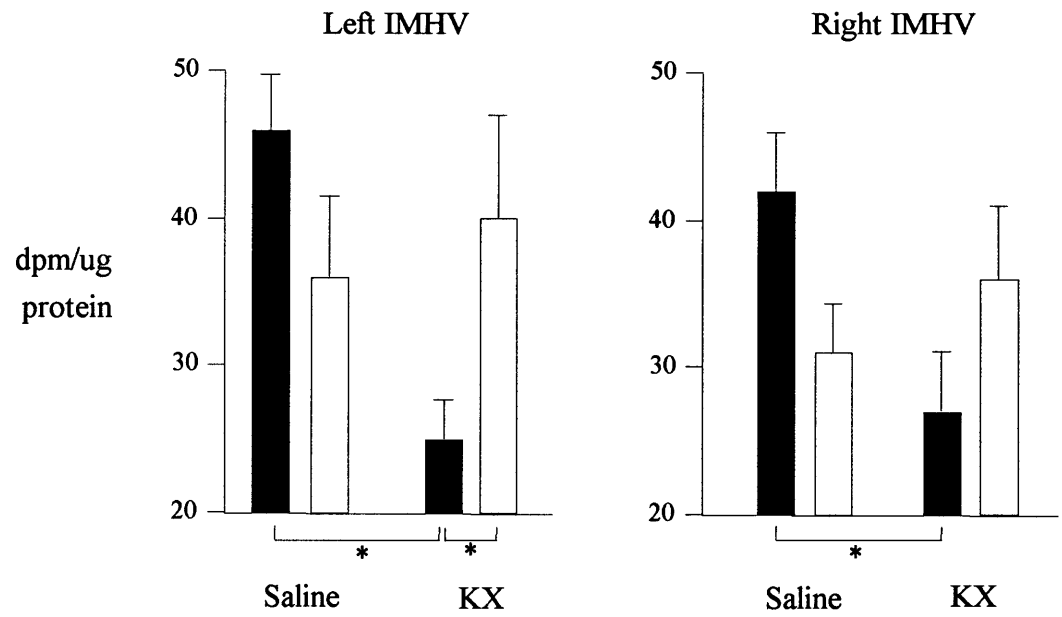
Individual t-tests were used to determine if there was a significant preference for the training stimulus in each of the groups. The difference between both KX-treated groups and the no-preference level of 50% was significant (box-trained group, $t = 3.49$, $p = 0.01$; hen-trained group, $t = 1.77$, $p = 0.05$, both one-tailed t-tests). The difference between both saline-treated groups and the no-preference level was not significant ($p > 0.05$ for both groups; two-tailed t-test).

4.3.4 [^{3}H]-MK-801 binding

Figure 4-3 presents the density of NMDA receptor binding, expressed in dpm/μg protein in the left and right IMHV for the four groups in this experiment. Left and right IMHV's were analysed separately using a two-factor analysis of variance. The factors were treatment (saline or KX) and visual experience (no visual experience or exposed to the imprinting stimulus).

For the samples of the left IMHV, there were no significant main effects of treatment ($F_{1,29} = 3.17$, $p = 0.09$) or visual experience ($F_{1,29} = 0.08$, $p = 0.78$). There was, however, a significant interaction between the factors treatment and visual experience ($F_{1,29} = 5.29$, $p = 0.03$). Accordingly, Fisher's LSD test was used to determine which groups contributed to this interaction. The binding in the group treated with KX but not exposed to the imprinting stimulus was significantly lower than the KX-treated group that had been exposed to the imprinting stimulus ($p < 0.05$) and the saline-treated group that did not receive any visual stimulation ($p < 0.025$).

In the right IMHV there were no significant main effects of treatment ($F_{1,29} = 1.66$, $p = 0.21$) or visual experience ($F_{1,29} = 0.01$, $p = 0.92$). There was, however, a significant interaction between the factors treatment and visual experience ($F_{1,29} = 5.49$, $p = 0.03$). Fisher's LSD test indicated that there was a significant difference between the two non-exposed groups. The KX treated group had significantly lower levels of binding compared to the saline-treated group ($p < 0.05$). Although none of the other groups



differed significantly there was a tendency for there to be an increase in density of NMDA receptors in the right IMHV of the imprinted chicks ($p < 0.10$).

Figure 4-3. Mean ± SEM density of NMDA receptors in the left and right IMHV of saline treated or KX-treated chicks.
The chicks were either exposed to the imprinting stimulus (clear bars) or were left in the dark (black bars) and hence were not exposed to the imprinting stimulus. The density of NMDA receptors in the left and right IMHV has been presented separately. The density of NMDA receptors is expressed as disintegrations per minute per μg protein. In the left IMHV binding was significantly lower in the KX treated chicks not exposed to the imprinting stimulus compared to KX treated chicks exposed to the imprinting stimulus and the saline treated chicks not exposed to the imprinting stimulus. In the right IMHV binding was also significantly lower in the KX-treated group not exposed to the imprinting stimulus and the unexposed saline-treated group.

Paired t-tests indicated that within each group, there were no significant differences between the left and right IMHV (two-tailed t-test, $p > 0.05$ for all groups).

Pearson rank order correlation co-efficients were calculated for the percent preference scores and the amount of [^{3}H]-MK-801 binding. The correlations were performed after combining data from the left and right IMHV for each pair of chicks as the analysis of the amount of [^{3}H]-MK-801 binding revealed no difference between the left and right IMHV. There was no significant correlation between the percent preference

scores and [^{3}H]-MK-801 binding in the IMHV for the overall data (r = 0.120, df = 9), nor were there significant correlations when the data was grouped by treatment (KX-treated, r = 0.121, df = 9 and saline-treated, r = - 0.054, df = 9).

4.4 Discussion

4.4.1 Imprinting

In the present experiment, the percent preference scores of the KX-treated chicks and the saline-treated chicks were not significantly different. However, the mean percent preference scores of both KX-treated groups trained on the hen and the box differed significantly from the no-preference level of 50%, while the mean percent preference score of the saline-treated chicks was not significantly greater than the no-preference level. Within each treatment group there was no difference between those chicks trained on the hen and those chicks trained on the box.

In the pilot experiment it was shown that chicks treated with a mixture of KX showed a significantly higher preference for the imprinting stimulus than untreated chicks only in the test 24 h after training. In the test 1 h after training there was no significant difference in the percent preference scores of the two groups. In the present experiment testing occurred 8 h after training and no significant difference in the percent preference scores of the two treatments was observed. The chicks were tested 8 h after training because a previous study using day 2 chicks has shown that NMDA receptor binding changes due to imprinting do not become evident until this time (McCabe and Horn, 1991).

When considering the results of the control (saline-treated) chicks of the present experiment with the untreated chicks of the pilot experiment, it appears as though the imprinting preferences of untreated chicks decreased during the period 1 to 24 h after training. It is possible that 8 h after training reflects an intermediate stage of the memory consolidation process in chicks of this age.

4.4.2 Binding results

The primary result of this chapter was that the density of NMDA receptors was significantly higher in the left IMHV of KX-treated chicks that had been imprinted compared to the left IMHV of the KX-treated chicks that remained in the dark. In contrast, no significant difference was found in the right IMHV of these animals although the tendency was for the same to occur. Thus, it would appear that imprinting on day 8 post-hatching uses similar neurochemical mechanisms to the imprinting which occurs during the peak sensitive period for imprinting (McCabe and Horn, 1988; 1991; Johnston *et al.*, 1993).

The studies using chicks aged 2 days post-hatching reported that there was an increased density of NMDA receptors in the left IMHV compared to the right IMHV (McCabe and Horn, 1988; 1991; Johnston et al., 1993). In the present experiment there was no significant difference in the density of NMDA receptors between the left and right IMHV of KX-treated chicks that were imprinted on day 8 post-hatching. McCabe and Horn (1988) found their effects in those chicks that had percent preference scores of between 80 and 100%. In the present study, the mean percent preference score of the KX-treated group was 71%. Had the number of chicks in the present experiment been greater it may have been possible to group the KX-treated chicks according to their percent preference score and determine if there is a difference in NMDA receptor density between the left and right IMHV in those chicks that had high percent preference scores.

Nevertheless, the density of NMDA receptors in the left IMHV of imprinted, KX-treated chicks was significantly greater compared to the KX-treated chicks that were left in the dark and not exposed to the imprinting stimulus. In contrast, in the saline-treated chicks exposure to the imprinting stimulus did not significantly affect the density of NMDA receptors. Actually, in both the left and right IMHV of saline-treated chicks, there was a tendency for the opposite to occur. The saline-treated chicks that were exposed to the imprinting stimulus tended to have slightly lower densities of NMDA receptors compared to the non-exposed saline-treated chicks. It is as if the NMDA receptors of

saline-treated chicks and KX-treated chicks respond to exposure to an imprinting stimulus in vastly different ways; NMDA receptors of KX-treated chicks increased in number, while NMDA receptors of saline-treated chicks showed a slight decrease. Furthermore, saline-treated chicks that were not exposed to the imprinting stimulus had significantly higher levels of binding compared to the non-exposed KX-treated chicks. It is as if two populations of NMDA receptors are present; one population found in KX-treated chicks, which increases in response to exposure to an imprinting stimulus, and another population found in saline-treated chicks which is unresponsive (or if anything the number of which declines) to exposure to an imprinting stimulus.

It is possible that KX treatment acts to modulate the ability to express a certain subtype of NMDA receptor that is important for the occurrence of activity-dependent events, such as imprinting. This receptor subtype may be endogenously expressed during the normal sensitive period for imprinting and its expression may have been altered by KX treatment. For example, Jakoi *et al.* (1992) have demonstrated that glutamate receptor activation produces long-lasting down regulation of gene expression in the hippocampus.

Different subtypes of NMDA receptors are known to be expressed at different stages of development, and it is thought that these subtypes of NMDA receptors may contribute to the ability or inability of synaptic changes to occur. For example, in the mammalian visual cortex, Carmignoto and Vicini (1992) have demonstrated an activity-dependent change in the excitatory post-synaptic current associated with the activation of the NMDA receptor that coincides with the sensitive period for plasticity in the visual cortex of the cat. Through an activity dependent mechanism the duration of the NMDA-stimulated post-synaptic current decreases, thus allowing less Ca^{2+} into the cell. Carmignoto and Vicini (1992) postulate that this change in the duration of the excitatory post-synaptic current is due to a subunit substitution in the NMDA receptor complex. Hestrin (1992) provides similar evidence from the superior colliculus of rats and proposes that a developmental regulation of the expression of NMDA receptors occurs. Both of these studies proposed that at different stages of development, different subtypes of NMDA

receptors with different molecular properties may be expressed, and that their expression is activity dependent. Thus, an experience-dependent mechanism for the control of synaptic plasticity involving the NMDA receptor is suggested.

The ending of the sensitive period for imprinting is thought to be an experience-dependent event (Bateson, 1979a). Chicks that have previously imprinted are less likely to form another imprinting memory. According to Bateson's Analysis Recognition Executive model (Bateson, 1981, 1991), the ability to learn the features of a new stimulus is not lost. Rather the ability of that particular stimulus to gain access to the executive system is lost and hence it cannot elicit the filial response. Bateson (1991) proposed that there is limited access from the recognition component of the system to the executive component of the system. Once the neural representation of a stimulus gains control of over half of the connections from the recognition component to the executive system it controls the filial response. Other stimuli are unable to gain control of the system because its capacity for plasticity is limited. The limited capacity for synaptic changes may be a reflection of the different properties of the NMDA receptor brought about by experience-dependent mechanisms. In the visual cortex it is thought that the expression of different subtypes of NMDA receptors may play a role in the control of plasticity (Carmignoto and Vicini, 1992; Hestrin, 1992).

Kavanaugh *et al.* (1991) have provided some evidence to indicate that there are two populations of NMDA receptors in the brain of the chick. One site was characterised as having a high affinity for [^{3}H]-MK-801 (K_D = 5.3 ± 0.5 nM), and the other site had a lower affinity for [^{3}H]-MK-801 (≈460 nM) but was not able to be as well characterised because of the range of ligand concentrations used. These values were obtained from chickens 11 days and 8-weeks-old, and no significant difference was found between the two age-groups. It would be very interesting to characterise the expression of NMDA receptors in the IMHV after imprinting both in day 2 chicks and in chicks 8-days old. Indeed, Johnston *et al.* (1993) have shown that as a result of imprinting there is an increase in affinity of L-glutamate receptors in day 2 chicks, and it is likely that this effect, like that of McCabe and Horn's (1988; 1991), is specific to the NMDA subtype of

glutamate receptor. There thus appears to be some evidence of a change in NMDA receptor subtype after imprinting.

4.5 Conclusion

Chicks treated with KX 10 h after hatching and subsequently exposed to an imprinting stimulus on day 8 post-hatching displayed a preference for the imprinting stimulus when tested 8 h after training. Saline-treated chicks did not show a preference for the imprinting stimulus.

While there was an increase in density of NMDA receptors in the left IMHV of KX-treated chicks that had been imprinted, there was no significant increase in the right IMHV, but there was a tendency for an increase. Comparisons were also made between KX-treated and saline-treated chicks that were dark-reared to day 8. The density of NMDA receptors in the left and right IMHV was significantly lower in the KX-treated chicks that had been dark-reared compared to the dark-reared saline-treated chicks. It appears that KX treatment 10 h post-hatching suppresses the number of NMDA receptors, but that in response to imprinting, NMDA receptor number in the left IMHV is able to significantly increase. It is proposed that KX treatment alters the expression of NMDA receptors that are normally associated with experience dependent synaptic changes. Through this or a similar mechanism chicks treated with KX are able to form an imprinting memory on day 8 post-hatching, at a time when this is not normally possible.

Chapter 5

A Sensitive Period for the Action of Ketamine-Xylazine

5.1 Introduction

The extension of the sensitive period for imprinting shown in the pilot experiment (see page 2) occurred as a result of a single injection of KX administered on the day after hatching. Using the same imprinting procedure on untreated chicks it was found that the sensitive period ended sometime between day 2 and 4 post-hatching (Chapter 3). Thus, the KX treatment was administered before the sensitive period for imprinting had ended. It is possible that the timing of the injection played an important role in the effect of the treatment. The previous Chapter indicated that imprinting which occurs on day 8 may have a similar cellular basis to imprinting which occurs on day 2. Thus, it is likely that in order for KX to be effective it must act before the sensitive period for imprinting has ended, and before any changes associated with the formation of an imprinting memory had occurred.

Therefore, in this experiment KX will be administered at varying post-hatch ages in order to determine if the age at which the chick receives its treatment has an effect on the ability to imprint on day 8 post-hatching.

5.2 Methods

One hundred and ninety one chicks, from 13 different hatches were tested in this experiment. Treatments were randomised throughout the hatches. The number of

chicks in each group and the stimulus on which they were trained are presented in Table 5-1.

During hatching the incubator was checked at least once every two hours, and any chicks that had hatched during this period were placed together in a separate compartment of the incubator. Chicks were randomly allocated to one of 10 groups consisting of chicks that received KX (55mg/kg ketamine and 6mg/kg xylazine, made up to a final volume of 0.1 ml by the addition of sterile pyrogen free, 0.9% saline) or they received 0.1 ml of the saline vehicle at one of the following post-hatching times: 10, 20, 40 h, 4 or 7 days. The chicks that were allocated to the 10, 20 or 40 h post-hatching groups were treated 9, 19 or 39 h after being transferred to the separate compartment. Thus, for these groups the injection time was accurate to within ± 1 h. The groups that received their treatment on day 4 or day 7 were treated mid-morning of the allotted treatment day. They had been removed from the incubator and placed in the group-rearing cages 20-24 h after hatching. As described on page 43, the chicks were dark-reared except when they were required for experimentation. Imprinting occurred on day 8 post-hatching according to procedures given on page 46.

5.3 Results

5.3.1 Activity in training

The total activity of the chicks during the training period is presented in Table 5-1. Activity was, however, measured as the number of revolutions towards or away from the imprinting stimulus. The logarithm of the activity was analysed using a four-factor analysis of variance with one repeated measure. The factors were treatment (saline or KX), age treated (10 h, 20 h, 40 h, 4 days or 7 days), stimulus (box or hen) and the repeated measure of direction of movement (towards or away from the imprinting stimulus).

Table 5-1. Total activity in training

Treatment		Time Treated				
		10 h	20 h	40 h	4 days	7 days
Saline	box	234 ± 93	261 ± 84	180 ± 80	118 ± 56	181 ± 39
	n	10	7	7	7	12
	hen	393 ± 169	750 ± 480	290 ± 98	325 ± 103	343 ± 176
	n	9	6	10	6	8
KX	box	381 ± 132	353 ± 88	306 ± 82	191 ± 74	289 ± 125
	n	8	9	11	11	14
	hen	512 ± 158	125 ± 48	285 ± 124	128 ± 34	421 ± 183
	n	11	15	13	9	8

The total activity of the chicks during the training period is presented as the mean ± SEM number of revolutions. Also presented is the number of chicks in each group.

There was a significant main effect of direction of movement ($F_{1,171}$ = 14.89, p = 0.0002). Significantly more activity was directed towards the imprinting stimulus than away from it. There were no significant main effects of treatment ($F_{1,171}$ = 0.14, p = 0.71), stimulus ($F_{1,171}$ = 1.22, p = 0.27) or age treated ($F_{1,171}$ = 0.55, p = 0.70). There were no significant interactions between any of the factors.

5.3.2 Activity in the preference tests

The total activity of the chicks in each of the preference tests was analysed separately. The activity in the test 1 h after training is presented in Table 5-2. The activity in the test 24 h after training is presented in Table 5-3. The data was log transformed and analysed using a three-factor analysis of variance. The factors were treatment (saline or KX) age treated (10 h, 20 h, 40 h, 4 days or 7 days), and stimulus (box or hen).

5.3.2.1 Test 1 hour after training

There were no significant main effects of treatment ($F_{1,104} = 0.96$, $p = 0.33$), age treated ($F_{4,104} = 1.71$, $p = 0.15$) or stimulus ($F_{1,104} = 0.64$, $p = 0.42$). There were no significant interactions between these factors.

Table 5-2. Total activity in preference test 1 hour after training

		Saline			KX		
Age treated	Stimulus	Reached activity criterion n	%	Total activity (revolutions)	Reached activity criterion n	%	Total activity (revolutions)
10 h	box	7	70	14.5 ± 7	6	75	15.6 ± 6
	hen	5	55	19.0 ± 12	7	64	45.0 ± 16
20 h	box	4	57	4.7 ± 4	5	56	2.8 ± 1
	hen	8	83	18.0 ± 9	10	67	17.0 ± 9
40 h	box	4	57	11.4 ± 8	5	45	1.4 ± 0.4
	hen	7	70	5.2 ± 2	10	77	17 ± 9
4 days	box	4	57	13.8 ± 6	7	64	4.0 ± 2
	hen	3	50	9.0 ± 3	5	56	2.0 ± 1
7 days	box	8	67	7.0 ± 4	10	71	7.9 ± 3
	hen	5	63	8.7 ± 5	7	88	9.1 ± 3

Mean ± SEM total activity in the preference test 1 h after training. Also presented is the number (n) and the percentage of chicks that reached the activity criterion in the test 1 h after training. There was no significant difference between the activity of the groups.

24 h after training (84%) compared to the test 1 h after training (65%). There were no significant interactions between the factors; treatment × stimulus ($X^2 = 5.47$, df = 9, $p > 0.10$), treatment × time ($X^2 = 3.23$, df = 9, $p > 0.10$) or time × stimulus ($X^2 = 0.20$, df = 1, $p > 0.10$).

5.3.3. Percent preference scores

The percent preference scores from each of the testing periods have been analysed separately. The scores from the test 1 h after training are presented in Figure 5-1. The scores from the test 24 h after training are presented in Figure 5-2. For each testing period the percent preference scores were transformed using the arcsine method (Winer, 1971) and analysed using a three-factor analysis of variance. The factors were treatment (saline or KX), age treated (10 h, 20 h, 40 h, 4 days or 7 days) and stimulus (box or hen). Only those chicks that reached the activity criterion were included in the analysis. For details of the activity criterion please refer to page 53. The percentage of chicks reaching the activity criterion in each of the testing periods is presented in Tables 5-2 and 5-3 respectively.

5.3.3.1 Test 1 hour after training

There were no significant main effects for any of the factors in the test 1 h after training (treatment, $F_{1,104} = 0.09$, $p = 0.77$; age treated, $F_{4,104} = 0.32$, $p = 0.86$; stimulus, $F_{1,104} = 2.59$, $p = 0.11$). There were no significant interactions between any of the factors (treatment × age treated, $F_{4,104} = 1.65$, $p = 0.17$; treatment × stimulus, $F_{1,104} = 0.01$, $p = 0.92$; age treated × stimulus, $F_{4,104} = 0.63$, $p = 0.65$; treatment × age treated × stimulus, $F_{4,104} = 0.99$, $p = 0.42$). It should be noted that the low number of animals reaching the activity criterion at this time could have contributed to the lack any significant effects.

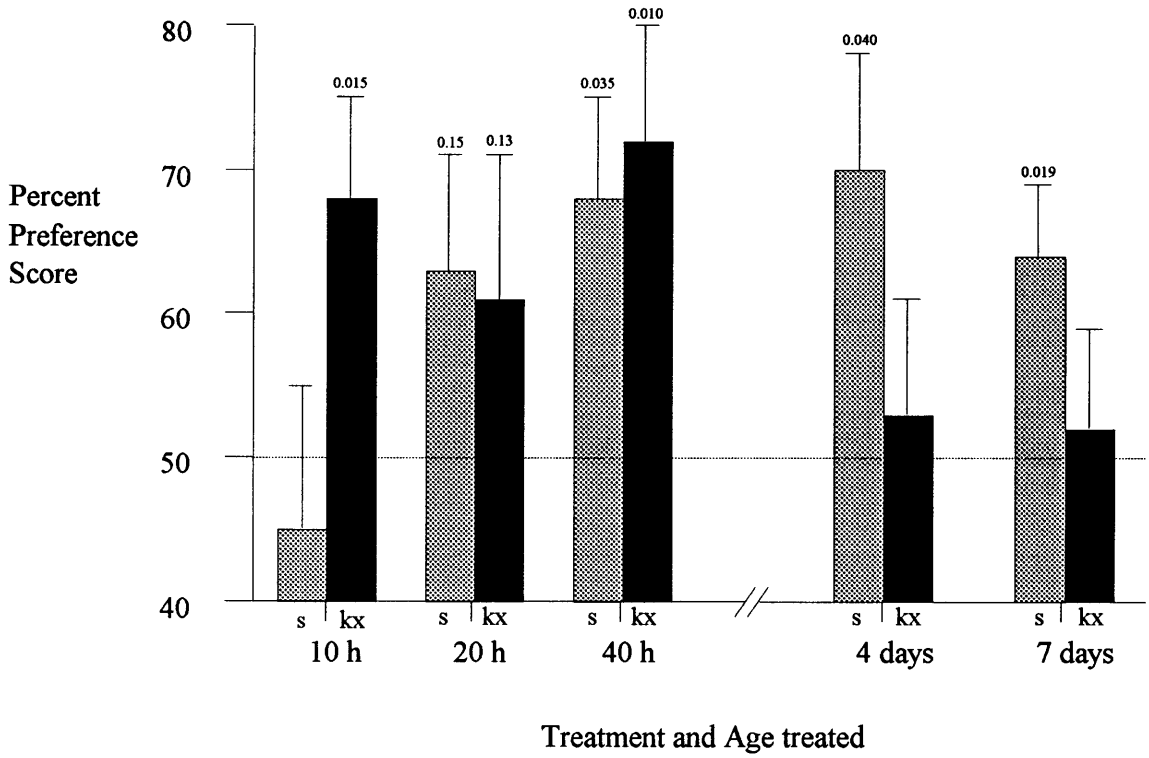


Figure 5-1. Mean ± SEM percent preference scores for the groups in the test 1 h after training. The data from the box and hen-trained groups have been combined. Grey bars represent saline-treated groups (box and hen trained), black bars represent KX-treated groups (box and hen trained). Overall, there was no significant difference in the percent preference scores of the groups. A number of groups did, however, show a significant preference for the imprinting stimulus. The levels of significance of these groups are indicated by the p values of t-tests applied between the scores of the groups and the no-preference level of 50%, which is presented above the error bars. The no-preference level is indicated by the dashed line.

Individual t-tests were used to determine if the mean preference score of a group differed significantly from the no-preference level of 50%. The results from the pilot experiment (see page 2) showed that the chicks treated with KX within the first 2 days post-hatching should show a preference for the imprinting stimulus. Therefore a one-tailed t-test was used. No such prediction was possible for the chicks treated with saline or the chicks treated with KX on day 4 or day 7, and a two-tailed test was used. The groups treated with KX at 10 ($p < 0.015$) and 40 h ($p < 0.010$) after hatching both

showed a significant preference for the imprinting stimulus, as did the groups treated with saline at 40 h ($p < 0.035$) 4 days ($p < 0.04$) and 7 days ($p < 0.019$). None of the other groups showed a significant preference for the imprinting stimulus.

5.3.3.2 Test 24 hours after training

The results from the test 24 h after training are presented in Figure 5-2. In the test 24 h after training there were no main effects of any of the factors (treatment, $F_{1,151} = 1.35$, $p = 0.25$; age treated, $F_{4,151} = 0.31$, $p = 0.87$; stimulus, $F_{1,151} = 0.48$, $p = 0.49$). There was, however, a significant interaction between the factors treatment and age treated ($F_{4,151} = 2.64$, $p = 0.04$). There was no significant interaction between the factors treatment and stimulus ($F_{1,151} = 0.01$, $p = 0.99$), age treated and stimulus ($F_{4,151} = 0.63$, $p = 0.65$) and no significant treatment × age treated × stimulus interaction ($F_{4,151} = 0.99$, $p = 0.42$).

Fisher's LSD tests were used to determine significant differences between the group means as defined by the significant interaction between the factors treatment and age treated. The group of chicks treated with KX 10 h post-hatching had significantly higher percent preference scores than the group of chicks treated with saline 10 h post-hatching ($p < 0.01$). There were no other significant differences between any of the KX-treated groups and the groups treated with saline at the same time. However, the group treated with KX at 10 h post-hatching also had significantly higher percent preference scores than the groups that were treated with KX on days 4 and 7 ($p < 0.01$ and $p < 0.05$ respectively). The group treated with KX at 10 h post-hatching also differed significantly from the group treated with saline at 20 h post-hatching. There were no other significant differences between any of the groups.

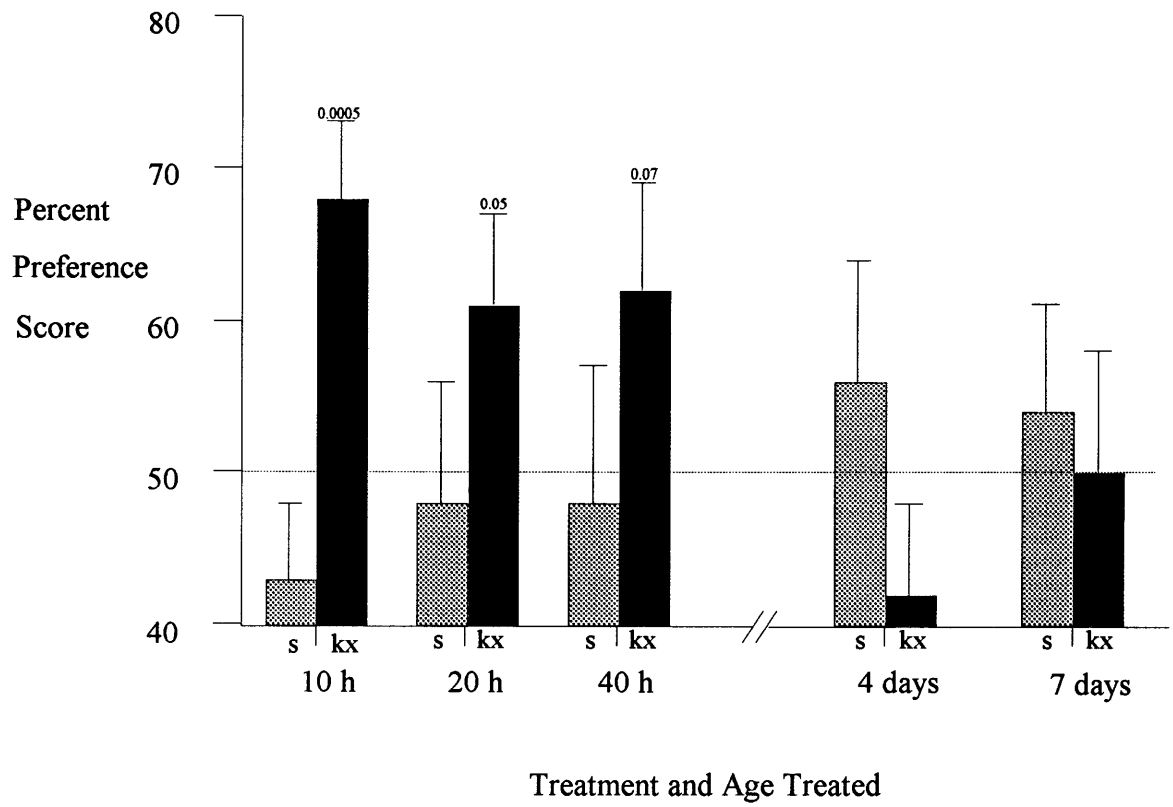


Figure 5-2. Mean ± SEM percent preference scores for the groups in the test 24 h after training. The data from the box and hen-trained groups have been combined. Grey bars represent saline-treated groups (box and hen trained), black bars represent KX-treated groups (box and hen trained). There was a significant interaction between the factors treatment and age treated. The preference of the groups treated with KX 10 and 20 h after hatching were significantly above the no-preference level of 50%. The levels of significance of these groups are indicated by the p values of t-tests applied between the scores of the groups and the no-preference level of 50%, which are presented above the error bars. The no-preference level is indicated by the dashed line.

Individual t-tests comparing the preference of the chicks to the no-preference level of 50% were performed. The p values are shown above the error bars on the figure. One-sided tests were only performed on the preference scores of the groups treated with KX at 10, 20 or 40 h after hatching. All other tests were performed using 2-sided tests. Only the groups treated with KX at 10 h or 20 h after hatching showed a significant preference (at $\alpha = 0.05$) for the imprinting stimulus.

When examining the mean group scores, categorised according to the stimulus that was used to imprint the chicks, it became apparent that the group of chicks treated with

KX at 10 h post-hatching and trained on the hen, had higher percent preference scores than the chicks trained on the box although there was no stimulus effect in the overall test. Therefore a post-hoc analysis of the percent preference scores, grouped according to the stimulus that was used to train the chicks was performed. These groups are presented in Figure 5-3.

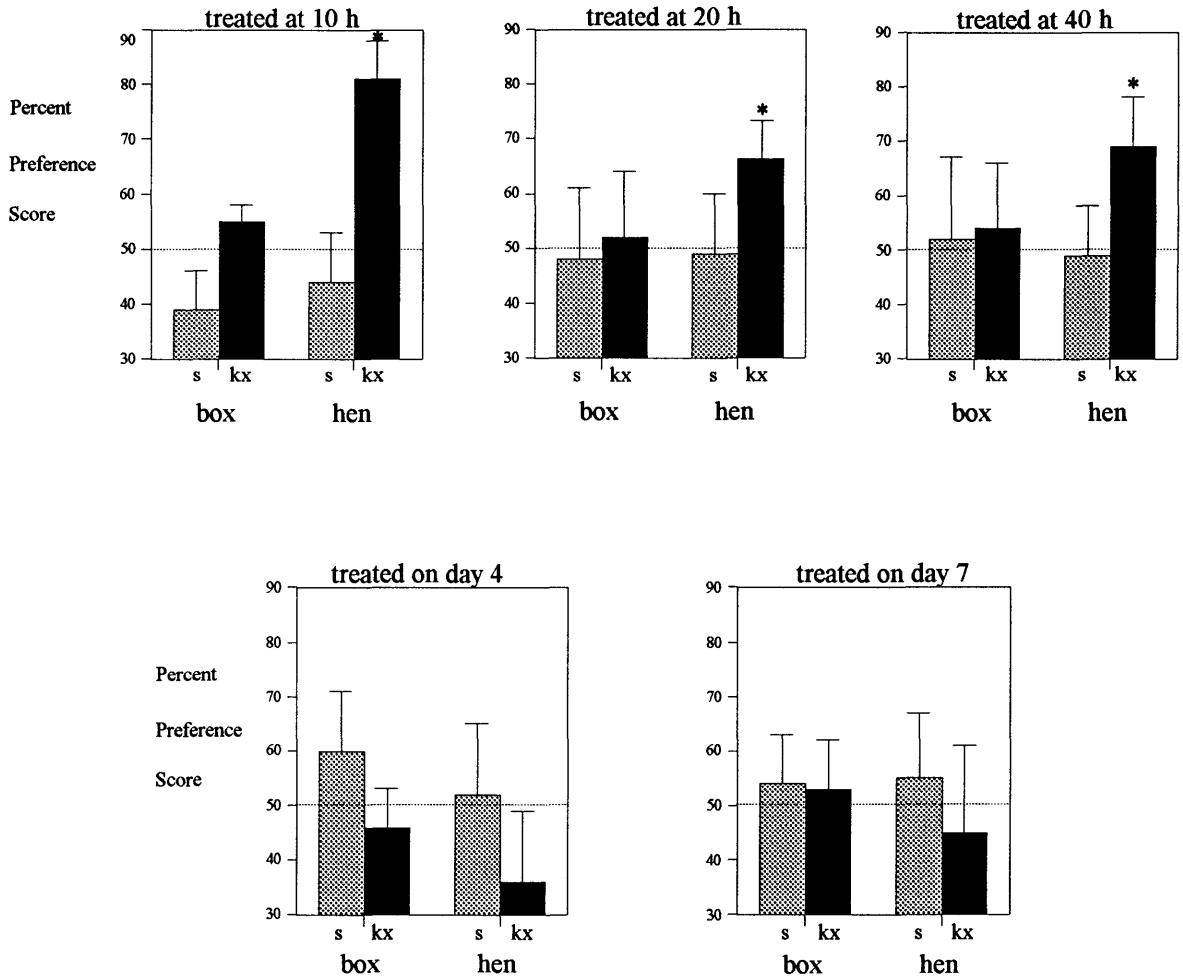


Figure 5-3. Percent preference scores plotted separately for the box and hen-trained chicks in the test 24 h after training.
The groups were treated with saline (grey bars) or KX (black bars) at five different ages. The group treated with KX 10 h post-hatching and trained on the hen had a significantly higher percent preference score than the other groups treated at this time. Additionally, the percent preference scores of the chicks treated with KX at 10 h, 20 h or 40 h post-hatching and trained on the hen were significantly higher than the no-preference level of 50%, which is indicated by the dashed line.

The group of chicks treated with KX at 10 h post-hatching and trained on the hen showed a significantly higher percent preference score than the chicks that received saline at 10 h post-hatching and which were trained on the hen ($p < 0.02$, two-tailed t-test). A comparison of the hen-trained chicks treated with KX at 10 h post-hatching with the chicks that received the same treatment, but which were trained on the box showed that the hen-trained chicks had significantly higher percent preference scores. One-tailed t-tests were used to compare the groups of chicks treated with KX at 10, 20 or 40 h after hatching and trained on the hen to the no-preference level of 50%. All three groups showed a significant preference for the hen at this testing period (KX 10 h, $p < 0.003$; KX 20 h, $p < 0.035$; KX 40 h $p < 0.04$).

5.4 Discussion

This experiment has confirmed the results of the pilot experiment which indicated that the sensitive period for imprinting could be extended by KX treatment early in life. In this experiment, those chicks that received an injection of KX 10 h or 20 h after hatching showed a significant preference for an imprinting stimulus 24 h after they were exposed to it on day 8 post-hatching. The group treated with KX 10 h after hatching showed a particularly high preference for the imprinting stimulus, while the preference of the group of chicks that received the injection of KX 40 h after hatching was not quite significant.

The analysis of variance of the percent preference scores in the test 24 h after training showed that there was no significant effect due to treatment alone. Rather, there was a significant interaction between treatment and the age that the chicks were treated. The group of chicks that were treated with KX 10 h after hatching showed a significantly higher preference for the imprinting stimulus than the chicks treated with saline 10 h after hatching. They also showed a significantly higher preference for the imprinting stimulus than the chicks treated with KX on days 4 or day 7, but were not significantly different to the groups treated with KX 20 or 40 h after hatching. However, it is clear that the effect

of KX treatment 20 h and 40 h after hatching is not as strong as the effect achieved by treatment with KX 10 h after hatching.

The percent preference score of the group of chicks treated with KX 40 h after hatching was not significantly different from the no-preference level of 50%. Additionally, the preferences of the chicks treated with KX 20 h or 40 h after hatching were not significantly different from the percent preference scores of the chicks treated with saline at these ages. In contrast, the preferences of the group of chicks treated with KX 10 h after hatching were significantly different from the chicks treated with saline 10 h after hatching. It thus appears as though the treatment is most effective 10 h after hatching, and declines thereafter.

There was an indication that the percent preference scores of the chicks treated with KX 10 h after hatching and trained on the hen were higher than the scores of the chicks treated with KX 10 h after hatching and trained on the box. KX treatment 10 h after hatching does not appear to have a significant effect on the ability of chicks to imprint on the box. This also appears to be the case for those chicks treated with KX 20 h or 40 h after hatching. The percent preference score of the group of chicks that were trained on the hen was significantly greater than the no-preference level of 50%, while the box-trained groups showed no such preference. However, it is important to remember that this effect was not shown in the full group analysis. It will be interesting to determine if the differential effect of the stimulus is also present in subsequent chapters.

The analysis of variance of the data from the test 1 h after training showed no significant difference between any of the groups for any of the factors. A number of groups did, however, show a significant preference for the imprinting stimulus. These were the groups treated with KX at 10 h or 40 h, but not 20 h after hatching, and the groups treated with saline at 40 h, 4 or 7 days after hatching. The preference shown by the groups at this time might be a reflection of a short-term memory. Furthermore, it should also be noted that fewer chicks reached the activity criterion in the test 1 h after training compared to the test 24 h after training. Thus, the results of the test 24 h after

training provide a better indication of the imprinting performance of the chicks, and this could have contributed to the lack of significance in the test 1 h after training.

The age of the chicks when they were treated with the mixture of KX undoubtedly plays an important role in the effect of extending the sensitive period. KX treatment is most effective when it is administered 10 h after hatching and is still effective when administered 20 h or 40 h after hatching. However, there is no effect of the treatment on days 4 or 7. Thus, there is a sensitive period during which the KX mixture is effective, which roughly coincides with the sensitive period for imprinting that was defined in Chapter 3 (which ends between days 2 and 4 post-hatching). At the very least, it can be said that the treatment is not effective after the sensitive period for imprinting has ended. It is likely therefore, that in order to mediate its effects the mixture of KX must act on the systems underlying imprinting before any changes associated with the ending of the sensitive period for imprinting may have occurred. The mixture of KX may do so by directly preventing an imprinting memory from forming, which seems unlikely because in doing so it would need to prevent imprinting for nearly a week, or it may have produced a long-term modulation on the systems underlying imprinting.

In the previous chapter it was shown that the density of NMDA receptors in the left IMHV of KX treated chicks increased significantly 8 h after imprinting. This suggests that the NMDA receptor is involved in the formation of an imprinting memory on day 8. The increase in NMDA receptor density in the IMHV of KX treated chicks may merely reflect the changes that occurred as a consequence of imprinting, and they do not necessarily indicate that the NMDA receptor is involved in mediating the extension of the sensitive period. However, the fact that ketamine is a component of the mixture of KX suggests that the NMDA receptor is involved in some way. By the same reasoning though, the noradrenergic system may also be involved. The following chapter will determine which components of the mixture of KX are involved in the extended sensitive period.

The effect on the extension of the sensitive period by KX treatment is specific to those chicks that were trained on the hen, and which were treated with KX within the first two days after-hatching. Imprinting did not occur in chicks that had been trained on

the box. There are two explanations for this; the hen may simply have been a more effective imprinting stimulus on day 8, or KX may not have extended the sensitive period for imprinting at all. Instead it may have affected the system that is responsible for the formation of a predisposition to approach a hen, without learning occurring at all (e.g. Bolhuis, 1991 and see page 18).

The predisposition for a chick to approach a hen occurs as a result of some rather non-specific stimulation during a discrete post-hatching period (sometime between 12 and 42 h post-hatching) (Johnson *et al.*, 1989). The exact nature of the stimulation needed to induce the predisposition is not known. Experimentally, the predisposition is induced by placing chicks of the appropriate age in imprinting wheels for a total of 2 h, although an operant training procedure has also been shown to induce the predisposition (Johnson *et al.*, 1985). The predisposition is not present 2 h after the experience in the wheels, but is present when chicks are tested 24 h later (Johnson *et al.*, 1985). Although the age of the chicks in the present experiment is well outside of the sensitive period for the development of the predisposition that was shown by Johnson *et al.* (1989), it is possible that the treatment could have extended or delayed the sensitive period for the development of the predisposition, as has recently been shown to occur by Davies *et al.* (1992) after treatment with the catecholaminergic neurotoxin DSP4 (see page 19 for further details). Davies *et al.* (1992) implicated the dopaminergic system in the developing predisposition because there was a negative correlation between the levels of dopamine in the IMHV and the percent preference scores for the fowl. However, the involvement of the dopaminergic system does not exclude the possibility that KX treatment influenced the sensitive period for the developing predisposition because there are known to be interactions between dopaminergic systems and the NMDA receptor system (Liljequist *et al.*, 1991). The possible interactions between the catecholaminergic systems and ketamine-xylazine treatment will be discussed in the next chapter which investigates the neurochemical mechanisms of the treatment.

An analysis of the training activity indicated that there was no significant difference between any of the groups. However, significantly more activity was directed towards

the imprinting stimulus than away from it during the training period showing that, regardless of the treatment of the chick, on day 8 chicks will show approach behaviour to a suitable stimulus. Thus, a stimulus can elicit approach behaviour from chicks without their necessarily forming a preference for that stimulus, inferring that the ability of a chick to form an imprinting memory is limited by the physiological processes underlying memory formation and not due to factors such as fear, which are thought to restrict the approach behaviour of chicks (Hess, 1959b).

5.5 Conclusion

The age at which a chick was treated with the KX mixture significantly affected its ability to imprint on day 8 post-hatching. Those groups that were treated with KX at 10, 20 or 40 h post-hatching were the only groups to show a preference for the imprinting stimulus. Thus, in order to be effective, KX treatment must be given before the sensitive period for imprinting has ended, or in other words, before any associated neural changes have occurred.

Additionally, only those chicks that were treated with KX at 10, 20 or 40 h and trained on the hen showed a significant preference for the imprinting stimulus in the test 24 h after training.

Chapter 6

Determining the Neurochemical Factors Involved in the Extension of the Sensitive Period by Ketamine-Xylazine Treatment

6.1 Introduction

This chapter is divided into three separate experiments. The first experiment examined the effect on the sensitive period for imprinting of administering single doses of ketamine (55 mg/kg), and single doses of xylazine (6 mg/kg) 10 h after hatching. The second experiment examined the effect of administering two doses of ketamine and two doses of xylazine in order to match the duration of KX anaesthesia. The third experiment examined the effect of administering the potent non-competitive NMDA receptor antagonist, MK-801.

Experiment 6-1

In the previous chapter chicks treated at 10, 20 or 40 h post-hatching with the mixture of KX and trained on the hen were shown to have a preference for it in the test 24 h after training. This effect is clearly due to an action of the mixture of KX since groups of chicks that received injections of saline at the same time showed no preference, regardless of the stimulus on which they were trained. The present experiment seeks to determine if the effect of the combination of KX can be achieved through the action of ketamine or xylazine alone. This experiment therefore investigates the individual roles that ketamine and xylazine may have in extending the sensitive period for imprinting.

6-1.1 Methods

One hundred and eight white leghorn × australorp cross chicks, from nine separate hatches were used in this experiment. Hatching and rearing conditions are described in Chapter 2 (see page 42). At 10 ± 1 h after hatching the chicks were treated with either the KX mixture (55 mg/kg ketamine and 6 mg/kg xylazine), ketamine alone (55 mg/kg), xylazine alone (6 mg/kg) or 0.1 ml of sterile pyrogen-free 0.9% saline. All treatments were given in a final volume of 0.1ml with the addition of sterile pyrogen-free 0.9% saline, and were injected into the gastrocnemius muscle. All handling of the chicks, including injections, was carried out in complete darkness. At no time did the chicks receive any visual stimulation.

In a pilot investigation, three groups of four chicks were given the same drug treatments and were observed in order to determine the duration of anaesthesia produced by KX, ketamine by itself or xylazine by itself. The mixture of KX produced a period of anaesthesia lasting for 50 ± 6 minutes, with full recovery 120-140 minutes after treatment. The duration of anaesthesia produced by ketamine alone was 20 ± 5 minutes, with full recovery occurring 80-100 minutes after treatment. Xylazine anaesthetised the chicks for 30 ± 7 minutes, and they were fully recovered by 100 minutes. Ketamine administered alone produced some distressing side-effects: 1) for the duration of the anaesthetic action the drug produced hypertonus which, when the chick regained consciousness was followed by a period of uncontrolled leg movement while the chick was on its side. This lasted for approximately 15 minutes and was followed by a period during which the chicks appeared to be sleeping; 2) 10 minutes later the chicks had righted themselves and began a period of uncontrolled locomotion. During this period, which lasted for about 10 minutes, they were unable to stand erect, but shuffled along the floor of the cage until they reached a side, whereupon they either collapsed or resumed movement in another direction.

The chicks were imprinted on day 8. Full details of the imprinting methods may be found in Chapter 2 on page 46. Briefly, the chicks were first primed and then placed in the imprinting wheels and exposed to either the rotating red and black box or a rotating stuffed hen for 2 h. They were then returned to the dark room where they had access to food and water. Two simultaneous preference tests were performed to determine if the chicks had formed an imprinting memory. The first test occurred 1 h after training, the second test on the following day, 20 ± 4 h later.

6-1.2 Results

6-1.2.1 Activity in training

The activity of the chicks towards and away from the imprinting stimulus during the training period is presented in Table 6-1. The data was log transformed and analysed using a 3 factor analysis of variance with a repeated measure on activity. The factors were treatment (saline, KX, ketamine or xylazine), stimulus (box or hen) and direction (towards or away from the imprinting stimulus). There were no significant main effects due to treatment ($F_{3,103} = 0.34$, $p = 0.80$) or stimulus ($F_{1,103} = 0.09$, $p = 0.76$). There was, however, a significant interaction between the factors treatment and stimulus ($F_{3,103}$

Table 6-1. Activity in training for chicks in experiment 6.1

Treatment	Stimulus	Number trained	Total activity (revolutions)	Activity towards (revolutions)	Activity away (revolutions)
Saline	box	14	398 ± 114	272 ± 106	126 ± 30
	hen	16	295 ± 65	171 ± 51	124 ± 38
KX	box	13	443 ± 127	243 ± 88	200 ± 95
	hen	17	275 ± 57	181 ± 47	94 ± 19
Ketamine	box	14	452 ± 140	346 ± 13	106 ± 20
	hen	10	281 ± 65	180 ± 56	101 ± 22
Xylazine	box	14	272 ± 56	136 ± 29	136 ± 29
	hen	12	614 ± 167	484 ± 171	130 ± 27

Table presents the mean ± SEM number of revolutions of the imprinting wheels during the training period. The number of revolutions are presented to the nearest complete revolution. The activity of the xylazine-treated group trained on the box is correct. Also presented is the number of chicks trained in each group.

= 2.70, p = 0.05). There was a significant main effect of the direction that the chicks moved ($F_{1,103}$ = 12.54, p = 0.001). Significantly more activity was directed towards the imprinting stimulus than away from it. There was no interaction between the factors direction and treatment ($F_{3,103}$ = 0.53, p = 0.66), nor was there an interaction between direction and stimulus ($F_{1,103}$ = 0.27, p = 0.60). The interaction between the factors direction, treatment and stimulus just failed to reach significance ($F_{3,103}$ = 2.51, p = 0.06).

Subsequent Fisher's LSD tests revealed that the interaction between the main effects of treatment and stimulus may be attributed to the xylazine treated group. In this group the chicks that were exposed to the hen had higher levels of activity than those chicks that were trained on the box ($p < 0.05$). There was no significant difference between the activity of the KX-treated groups that were trained on the box or the hen.

6-1.2.2 Activity in the preference tests

The mean total activity of the groups in both preference tests is presented in Table 6-2. Only those chicks that reached the activity criterion were included in this analysis. The data from the tests 1 h after training and the test 24 h after training were analysed separately. The log of the activity was analysed using a two-factor analysis of variance.

In the test 1 h after training there were no main effects of treatment ($F_{3,49}$ = 0.51, p = 0.68) or stimulus ($F_{1,49}$ = 0.06, p = 0.80). There were no interactions between these factors.

In the test 24 h after training there were no main effects of treatment ($F_{3,78}$ = 1.10, p = 0.68) or stimulus ($F_{1,78}$ = 0.12, p = 0.73). There was, however, a significant interaction between these factors ($F_{3,78}$ = 3.58, p = 0.02). Fisher's LSD test showed that the KX-treated group trained on the hen had significantly lower activity than all other groups that were trained on the hen ($p < 0.05$) and the KX treated group trained on the box ($p < 0.01$). The only other significant difference was between the group treated with ketamine and trained on the box and the saline-treated group that was trained on the box ($p < 0.01$).

Table 6-2. Mean total activity in preference tests.

Treatment	Stimulus	Test at 1 hour			Test at 24 h		
		Reached activity criterion		Total activity (revolutions)	Reached activity criterion		Total activity (revolutions)
		n	%		n	%	
Saline	box	5	36	4.3 ± 3	10	71	5.8 ± 3
	hen	9	56	7.5 ± 3	15	94	8.7 ± 3
KX	box	7	54	10.9 ± 6	9	69	12.3 ± 5
	hen	8	47	5.0 ± 3	12	71	3.5 ± 7
Ketamine	box	7	50	14.6 ± 6	12	86	15.0 ± 6
	hen	4	40	12.0 ± 7	7	70	15.8 ± 6
Xylazine	box	9	64	3.9 ± 1	11	79	6.3 ± 3
	hen	8	67	6.8 ± 3	10	83	13.6 ± 5

Mean ± SEM activity of the chicks during the testing periods. Activity was measured in revolutions of the imprinting wheels. Also presented is the number (n) and percentage of chicks that reached the activity criterion in each of the testing periods.

6-1.2.2.1 Number of chicks that reached the activity criterion

The proportion of chicks reaching the activity criterion was analysed using the weighted least squares approach that is described on page 55. The main effect of time tested was significant ($X^2 = 18.39$, df = 1, $p < 0.001$). Overall, significantly more chicks reached the activity criterion in the test 24 h after training (78%) than in the test 1 h after training (52%). The main effects of treatment ($X^2 = 2.47$, df = 3, $p > 0.10$) and stimulus ($X^2 = 0.07$, df = 1, $p > 0.10$) were not significant. There were no significant interactions between any of the factors; treatment × stimulus ($X^2 = 4.80$, df = 3, $p > 0.10$), treatment × time tested ($X^2 = 2.48$, df = 3, $p > 0.90$), stimulus × time tested ($X^2 = 0.06$, df = 1, $p > 0.90$).

6-1.2.3 Percent preference scores

The percent preference scores for the test 1 h after training are presented in Figure 6-1. the percent preference scores from the test 24 h after training are presented in Figure 6-2. The data was transformed by the arcsine method (Winer, 1971) and analysed using a 2 factor analysis of variance. The factors were treatment and stimulus used in training. T-tests on the arcsine transformed data were performed to determine which groups showed a significant preference for the imprinting stimulus.

6-1.2.3.a Test 1 hour after training

In the test 1 h after training (Fig. 6-1) there was no significant main effect due to treatment ($F_{3,49} = 1.57$, $p = 0.21$) or stimulus ($F_{1,49} = 0.12$, $p = 0.73$), nor was there an interaction between the factors ($F_{3,49} = 0.95$, $p = 0.42$). Both the box trained ($t = 3.05$, $p < 0.01$, one-tailed t-test) and the hen trained ($t = 2.24$, $p < 0.03$, one-tailed t-test) KX-treated groups showed a significantly higher preference for the imprinting stimulus than the no-preference level of 50%. None of the other groups, including the box trained ketamine-treated group ($t = 2.85$, $p = 0.07$, two-tailed t-test) showed a significant preference for the imprinting stimulus.

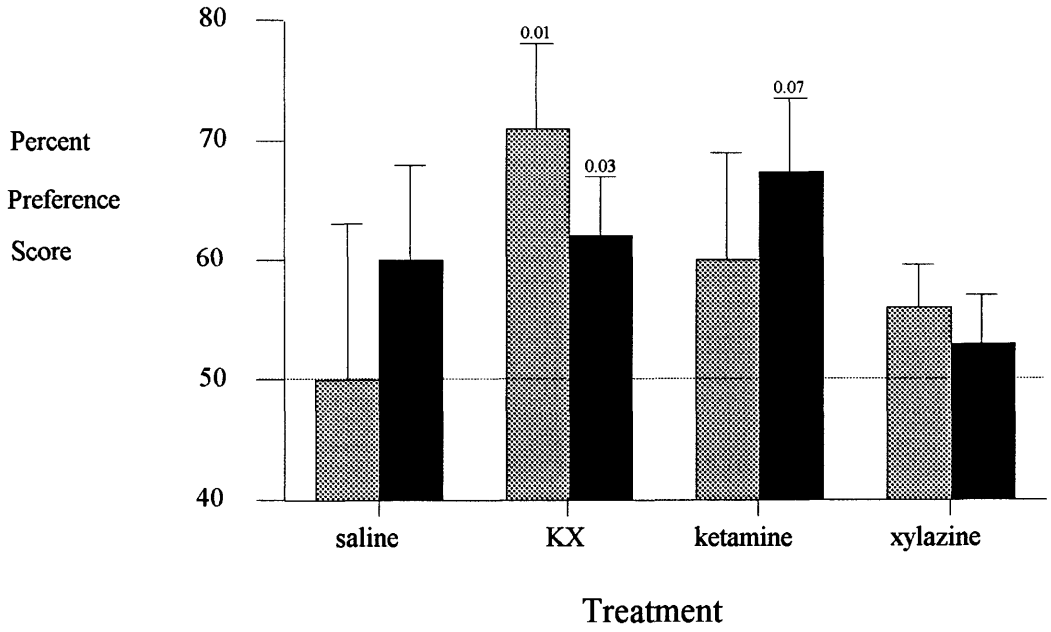


Figure 6-1. Mean ± SEM percent preference scores in the imprinting test 1 h after training. Grey bars represent the box-trained groups, black bars represent the hen-trained groups. Both KX-treated groups showed a significant preference for the imprinting stimulus. None of the other groups showed a significant preference for the imprinting stimulus. The dashed line indicates the no-preference level of 50 %.

6-1.2.3.b Test 24 hours after training

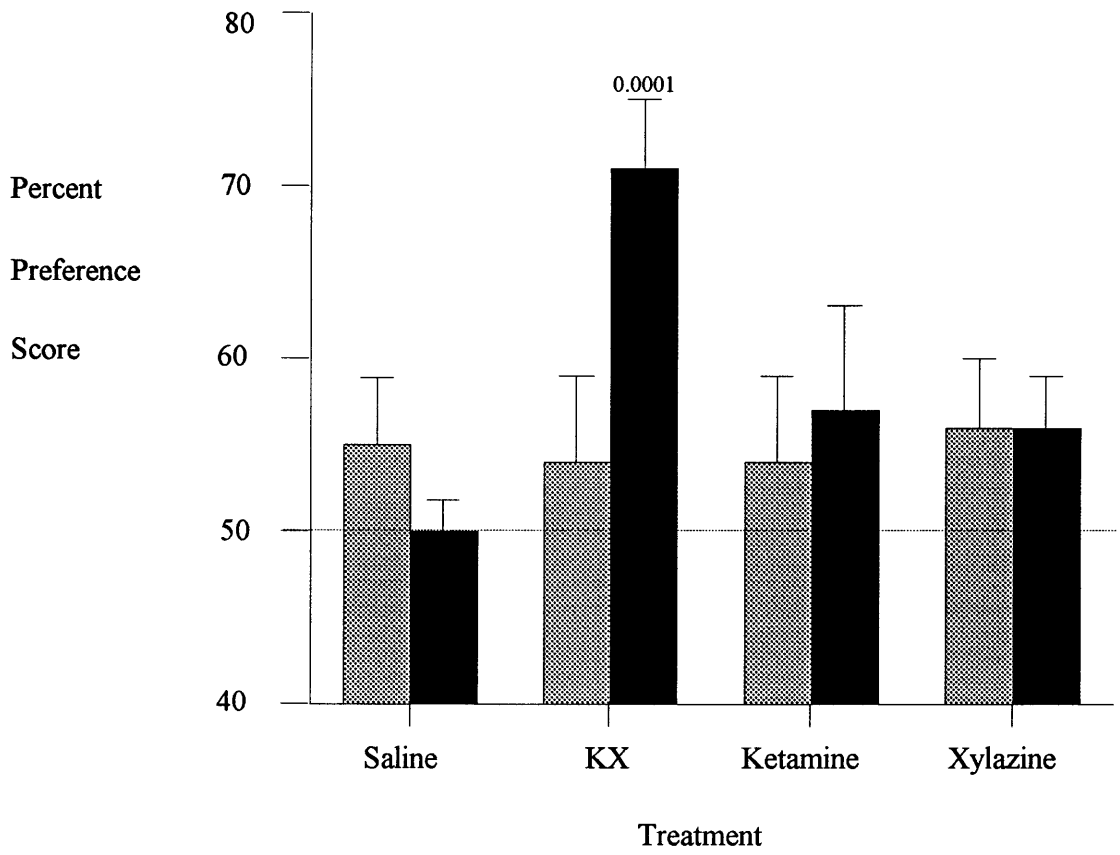


Figure 6-2. Mean ± SEM percent preference scores in the imprinting test 24 h after training. Grey bars represent the box-trained groups, black bars represent the hen-trained groups. Only the KX-treated group that was trained on the hen showed a significant preference for the imprinting stimulus. The dashed line represents the no-preference level of 50%.

In the test 24 h after training (Fig. 6-2) there was no significant main effect due to treatment ($F_{3,78} = 2.24$, $p = 0.07$) or stimulus ($F_{1,78} = 2.18$, $p = 0.14$), but there was a significant interaction between the factors treatment and stimulus ($F_{3,78} = 2.67$, $p = 0.05$). Fisher's LSD tests were used to make individual comparisons between the means. The group treated with KX and trained on the hen had a significantly higher percent preference score than the other groups trained on the hen and the KX-treated group trained on the box ($p < 0.05$). The KX-treated group that was trained on the hen was the only group that showed a significant preference for the imprinting stimulus ($t = 4.67$, $p < 0.0001$, one-tailed t-test).

6-1.4 Discussion

The main result of this experiment is that chicks treated with either ketamine or xylazine alone were unable to form a preference for an imprinting stimulus on day 8 post-hatching, as evidenced by a lack of preference shown in the test 24 h after training. This was true regardless of the stimulus that was used to train the chicks. This experiment also confirmed the results of the previous chapter in that the group of chicks that were treated with the mixture of KX 10 h after hatching and trained on the hen were able to form a preference for it in the test 24 h after training. No such preference was shown by the KX-treated group trained on the box or the saline-treated group trained on either of the stimuli. Thus, the stimulus used to train the chicks has an influence on whether imprinting will occur on day 8. The importance of the stimulus was also noted in the previous chapter where possible reasons for the chicks imprinting only on the hen were discussed. This matter is further discussed in Chapter 7.

The results from this experiment suggest that ketamine or xylazine alone do not produce the same effect as the mixture of KX; that is, they do not enable chicks to form a filial preference in the second week of their life. Instead, the ability to form a filial preference must be due to a combined action of the drugs. These results must be qualified to include the fact that, at the doses used, the drugs do not produce the same duration of anaesthesia. The duration of anaesthesia produced by the mixture of KX is approximately twice that produced by ketamine or xylazine alone. While not suggesting that it is an anaesthetic property of the drug that is producing the effect, an indication of the length of their bioactivity may be obtained from the length of anaesthesia. Certainly, the length of anaesthesia produced by the combination of KX indicates a synergistic action of the KX mixture.

As in the previous chapter, the results of the first imprinting test, which occurred 1 h after training, were ambiguous. No significant variation in percent preference scores was shown at this time, although it was only the groups that were treated with KX that

showed a significant preference for the imprinting stimulus. The variability of the results in the test 1 h after training in comparison to the rather uniform results shown in the test 24 h after training might be explained by the temporal proximity of the 1 h test in relation to the training period. At 1 h after training the stimulus is fresh in the experience of the chicks and recall may not be reliant on a long-term memory, while 24 h after training recall may depend on a long-term memory. It may also be noted that fewer chicks contributed to the scores in the test 1 h after training than in the test 24 h after training.

The activity in training proved interesting in that there was an interaction between the main effects of treatment and stimulus. The main effects analysis takes an average of the two repeated measures; this is therefore a measurement of the total activity of the chicks. The activity of the ketamine-treated chicks during the training period was not significantly different to the activity of the saline-treated chicks. Ketamine thus had no effect on the activity of the chicks during training. In both the ketamine and the saline-treated groups, those chicks trained on the box were significantly more active than those chicks trained on the hen. There was no significant difference in the activity of the box-trained versus the hen-trained KX-treated groups. Thus, during the training period differences in the activity of the chicks have been shown to occur which are dependent upon both the stimulus that is presented and the treatment of the chicks.

Based on the activity during training, the hen appears to elicit more *following* behaviour than the box in xylazine-treated chicks. This is curious as the noradrenergic system is not thought to be involved in the filial response towards the hen (Davies *et al.*, 1985, 1992). However, this interesting effect of xylazine is not reflected in the percent preference scores of the chicks, despite their level of activity during the training and testing periods.

There were no significant differences in the activity of the groups in the test 1 h after training. In the test 24 h after training the activity of the hen-trained KX-treated group was significantly lower than the other hen-trained groups and the box-trained KX-treated group. It is as if the activity of the hen-trained KX-treated group had been suppressed during this test, which may be expected if the chicks were overly fearful. A heightened

level of fearfulness has classically been observed to coincide with imprinting (e.g. Hess, 1959c; Bateson, 1964c and see page 20). It is possible that the lower activity of the imprinted chicks is due to their fearful state induced by the alternative stimulus, although one may have expected them to run away from the alternative stimulus and move towards the familiar stimulus. In fact, the chicks did move towards the familiar stimulus as evidenced by their preference scores, but their activity was low.

The results from this experiment indicate that, at the doses used, neither ketamine or xylazine are capable of producing the same effect as the mixture of KX.

Experiment 6-2

This experiment investigated whether the duration of action of the drugs (as measured by the duration of the anaesthetic action) was a factor in the lack of effectiveness of the groups treated with ketamine alone or xylazine alone in the previous experiment. Ketamine (55 mg/kg) or xylazine (6 mg/kg) produced periods of anaesthesia which were somewhat less than half as long as the period of anaesthesia that was produced when these drugs were given in combination. It is thus possible that the lack of effect shown by the chicks that were treated with ketamine alone or xylazine alone is due to the fact that these drugs were not acting for a long enough period. In other words, there may be a minimum period during which the treatments must act in order to have an effect on the imprinting system. Therefore, in this experiment chicks received two injections of ketamine and two injections of xylazine in order to increase the duration of action of these drugs.

6-2.1 Methods

A total of 103 chicks from eight separate hatches were used in this experiment. Incubation and rearing conditions were as described in Chapter 2 (see page 42). All handling prior to the imprinting procedures, including injecting the chicks, was done in

complete darkness. Treatments were randomised across the hatches. The number of chicks trained in each group is presented in Table 6-3. The number of chicks reaching the activity criterion at each testing period is presented in Table 6-4. In order to increase the period of exposure to ketamine and xylazine each of these separate treatments were given twice, without altering the dose that was used in the previous experiment. The first treatment was given 10 h after hatching, and the second treatment followed 2 h later. This ensured that the initial concentration of the drugs was not greater than that which was given in the mixture of KX. The group treated with ketamine twice is referred to as the ket. × 2 group and the group treated with xylazine twice is referred to as the xyl. × 2 group. Another group received the KX mixture, and another group was treated with 0.9% sterile, non-pyrogenic saline. The chicks were dark-reared to day 8, then imprinted according to the method on page 46.

6-2.2 Results

6-2.2.1 Activity in training

The mean ± SEM activity of the chicks towards and away from the imprinting stimulus during the training period is presented in Table 6-3.

The activity in training (measured in revolutions of the wheels) was log transformed and analysed using a three-factor analysis of variance with one repeated measure. The factors were treatment (saline, KX, ket. × 2 and xyl. × 2), stimulus (box or hen) and direction of movement (towards or away from the imprinting stimulus). There was a significant main effect of direction ($F_{1,95}$ = 12.16, p = 0.001), with significantly more activity being directed towards the imprinting stimulus. There was no significant main effect of treatment ($F_{3,95}$ = 1.64, p = 0.19) or stimulus ($F_{1,95}$ = 0.60, p = 0.44). There were no significant interactions between any of the factors although the interaction between treatment and stimulus ($F_{3,95}$ = 2.46, p = 0.07) and direction and stimulus ($F_{1,95}$ = 3.66, p = 0.06) were very close to reaching statistical significance.

Table 6-3. Activity of chicks during training.

Group	Stimulus	n	Total activity (revolutions)	Activity towards (revolutions)	Activity away (revolutions)
Saline	box	16	415 ± 92	224 ± 65	190 ± 53
	hen	11	216 ± 69	116 ± 38	99 ± 31
KX	box	13	296 ± 114	145 ± 46	150 ± 72
	hen	15	394 ± 103	283 ± 92	111 ± 28
Ket. × 2	box	12	309 ± 68	191 ± 53	118 ± 23
	hen	14	385 ± 94	288 ± 92	97 ± 20
Xyl. × 2	box	10	372 ± 64	196 ± 32	176 ± 35
	hen	12	551 ± 178	475 ± 182	76 ± 17

Mean ± SEM activity in training. Activity was measured in revolutions of the imprinting wheels. Training scores were not available for 1 ket. × 2 (hen) chick and 1 xyl. × 2 (hen) chick.

6-2.2.2 Activity in the preference tests

The total activity in the preference tests 1 h and 24 h after training is presented in Table 6-4. The data from each testing period was analysed separately using a two-factor analysis of variance on the log-transformed data.

In the test 1 h after training there were no main effects of treatment ($F_{3,53} = 0.45$, $p = 0.72$) or stimulus ($F_{1,53} = 3.68$, $p = 0.06$). There was no significant interaction between the factors ($F_{3,53} = 0.95$, $p = 0.43$).

Similar results were also obtained in the test 24 h after training. There was no significant effect of treatment ($F_{3,77} = 2.02$, $p = 0.12$) or stimulus ($F_{1,77} = 3.23$, $p = 0.08$). There was no significant interaction between the factors ($F_{3,77} = 1.15$, $p = 0.33$).

Table 6-4. Activity in the preference tests.

Treatment	Stimulus	Test 1 h after training			Test 24 h after training		
		Reached activity criterion		Total activity (revolutions)	Reached activity criterion		total activity (revolutions)
		n	%		n	%	
Saline	box	10	63	3.6 ± 1.3	15	94	2.7 ± 0.8
	hen	5	45	4.1 ± 2.5	10	91	4.5 ± 2.3
KX	box	2	15	0.3 ± *	8	62	0.5 ± 0.2
	hen	6	40	4.8 ± 1.8	13	87	4.6 ± 1.5
Ket. × 2	box	8	67	2.5 ± 0.8	10	83	7.8 ± 4.1
	hen	9	67	9.1 ± 6	12	87	4.8 ± 2.1
Xyl. × 2	box	7	70	3.0 ± 1.5	9	90	3.6 ± 1.5
	hen	11	77	9.6 ± 7.3	7	69	12.2 ± 6.9

Activity is presented as the mean ± SEM number of revolutions in the tests 1 h and 24 h after training. Also presented is the number (n) and percentage of chicks that reached the activity criterion in each of the testing periods.

6-2.2.3 Number of chicks that reached the activity criterion

The proportion of chicks reaching the activity criterion is presented in Table 6-4. The data was analysed using the weighted least squares method that is described on page 55. The main effect of treatment was significant ($X^2 = 17.45$, df = 3, $p < 0.001$), and is clearly due to the KX-treated group in which in total only 51% of the chicks reached the activity criterion compared to the saline-treated group (73%), the ket. × 2-treated group (76%) and the xyl. × 2-group (76%). The main effect of time tested was also significant ($X^2 = 16.99$, df = 1, $p < 0.001$), with fewer chicks reaching the activity criterion in the test 1 h after training (56%) than in the test 24 h after training (83%). The main effect of stimulus ($X^2 = 0.65$, df = 1, $p > 0.10$) was not significant. There was a significant interaction between the factors treatment and time tested ($X^2 = 12.61$, df = 3, $p < 0.01$). There were no significant interactions between any of the other factors; treatment ×

stimulus ($X^2 = 4.57$, df = 3, $p > 0.10$) or stimulus × time tested ($X^2 = 0.40$, df = 1, $p > 0.90$).

6-2.2.4 Percent preference scores

6-2.2.4.a Test 1 hour after training

The results from the test 1 h after training are presented in Table 6-5. There were no significant main effects for treatment ($F_{3,53} = 2.40$, $p = 0.08$) or stimulus ($F_{1,53} = 3.09$, $p = 0.09$), and no interaction between these factors ($F_{3,53} = 0.23$, $p = 0.88$). As previously mentioned, the test at this period is notable in that only two of the KX-treated chicks trained on the box reached the activity criterion. Only the box-trained ket. × 2-treated group showed a significant preference for the imprinting stimulus ($p < 0.05$, two-tailed t-test).

Table 6-5. Percent preference scores in test 1 h after training

Treatment	Stimulus	Percent preference score ± SEM
Saline	box	40 ± 10
	hen	48 ± 18
KX	box	75 ± *
	hen	81 ± 16
Ket. × 2	box	55 ± 10
	hen	84 ± 10
Xyl. × 2	box	62 ± 15
	hen	75 ± 11

Mean ± SEM percent preference scores in the test 1 h after training are presented in the table. There were no significant differences between the groups, although only the ket. × 2 group trained on the hen showed a significant preference for the imprinting stimulus. * only two chicks reached the activity criterion in the KX box-trained group.

6-2.2.4.b Test 24 hours after training

The results from the test 24 h after training are presented in Figure 6-3. Only the hen-trained, KX-treated group ($p < 0.0005$, one-tailed t-test) and the hen-trained ketamine treated group ($p < 0.003$, two-tailed t-test) showed a significant preference for the imprinting stimulus.

There were significant main effects of treatment ($F_{3,77} = 2.81$, $p = 0.05$) and stimulus ($F_{1,77} = 6.71$, $p = 0.01$). Overall, those chicks trained on the hen had higher percent preference scores. There was no significant interaction between the factors treatment and stimulus ($F_{3,77} = 0.96$, $p = 0.41$).

Fisher's LSD tests on the treatment means showed that the KX-treated group and the group treated twice with ketamine differed significantly from the group treated twice with xylazine ($p < 0.05$). The difference between these groups and the saline-treated group was not significant. However, it is clear that the preference scores of the box-trained chicks pulls the mean percent preference score of the groups down.

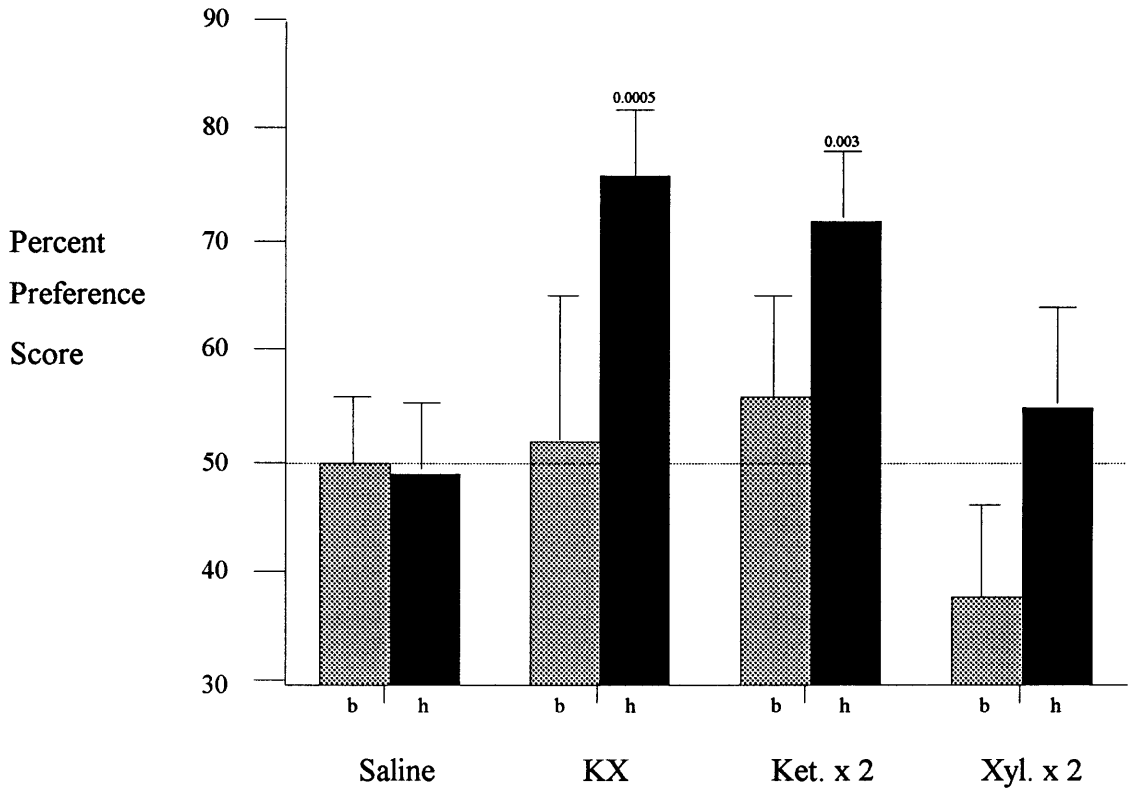


Figure 6-3. The mean ± SEM percent preference scores in the test 24 h after training.
b: box-trained group, h: hen-trained. The dashed line represents the no-preference level of 50%. Only the groups of chicks treated with KX or ket. × 2 and trained on the hen showed a preference that was significantly greater than the no-preference level of 50%. The level of significance is indicated by the p-values presented above the error bars.

6-2.3 Discussion

In contrast to Experiment 1 of this chapter, this experiment showed that treatment with ketamine alone is capable of extending the sensitive period for imprinting. Coupled with the results of the previous experiment, it is apparent that the duration of the treatment is important. Chicks treated with a single dose of ketamine (55 mg/kg) 10 h after hatching showed no extension of the sensitive period. In the present experiment, chicks received the same injection of ketamine 10 h after hatching and another dose 2 h later. Chicks treated in this manner and exposed to a hen for a 2 h period on day 8 showed a preference for the hen in the test 24 h after training. No preference was

evident in chicks that were trained on the box. As in the previous experiments, chicks treated with KX imprinted on the hen on day 8 post-hatching, but KX treated chicks trained on the box and saline treated chicks trained on the box or the hen failed to show a preference for the imprinting stimulus. Chicks that were treated with xylazine at 10 h and 12 h post-hatching showed no evidence of imprinting on either the hen or the box. Indeed, xylazine inadvertently proved to be a valuable control treatment in that it produced no effect on the ability to imprint on day 8, but produced a comparable level of anaesthesia.

The similarity between the results of the KX-treated group and the group that received the two doses of ketamine in the test 24 h after training is striking. Within these groups, those chicks that were trained on the hen showed a significant preference for it in the test 24 h after training. No preference was shown by those chicks trained on the box. Thus, it appears as though the ketamine component of the mixture of KX mediates the effect on the imprinting system.

The fact remains, however, that there is an interaction between ketamine and xylazine. A single 55 mg/kg dose of ketamine does not allow chicks to imprint on day 8 post-hatching unless it is combined with xylazine. It has been shown that the length of time that the mixture of KX acted is approximately twice as long as when ketamine or xylazine act when given alone. The purpose of this experiment was to adjust the length of time that the chicks were exposed to the actions of ketamine and xylazine (given separately) so that it was equal to the duration of anaesthesia caused by KX. When this was done, the group of chicks that received the two doses of ketamine imprinted on the hen on day 8 post-hatching, much the same as the group treated with the mixture of KX. Non-specific anaesthetic effects can be ruled out because the chicks that received two doses of xylazine were anaesthetised for a similar period of time to the ketamine treated chicks, yet showed no imprinting preference. Thus, in KX treated chicks the effect of extending the sensitive period is probably due to the ketamine component of the mixture.

Undoubtedly, xylazine plays an indirect role in the action of ketamine when the two drugs are administered together. This role may simply be related to the extra period of

time that ketamine is able to act when it is in the presence of xylazine. Although, xylazine is commonly given with ketamine for its centrally induced muscle relaxant properties (Wright, 1982), α2-adrenergic agonists also have the capacity to prolong the period during which ketamine is available through inhibiting the hepatic biotransformation of ketamine (Kharasch *et al.*, 1992). Acknowledging that species differences in the biotransformation of ketamine may exist, xylazine, by inhibiting the breakdown of ketamine, could essentially have acted to extend the duration of ketamine action.

As has been noted in previous experiments, fewer chicks reached the activity criterion in the test 1 h after training compared to the number reaching the activity criterion in the test 24 h after training. In this experiment, this was particularly evident in the KX treated chicks that were trained on the box. In this group only two chicks reached the activity criterion in the test 1 h after training. An exceptionally low number of chicks that reached the activity criterion in the test 1 h after training has not been observed in any of the previous experiments.

The activity levels of the groups did not differ significantly in the training period or in either of the testing periods. Thus, the treatments did not significantly alter the activity of the animals. The effect shown by the treatment on the percent preference scores can not be attributed to one group of chicks being more active in response to a stimulus. Indeed, as a later analysis will show (see page 160), the activity of these chicks during the testing periods is positively correlated with their activity in an open field.

In summary, this experiment has provided a clear indication that the ketamine component of the mixture of ketamine and xylazine is responsible for the effect of extending the sensitive period for imprinting.

Experiment 6-3

It is probable that ketamine mediates its effect on the sensitive period for imprinting through its action as a non-competitive antagonist of the NMDA receptor (Thomson *et*

al., 1985; Anis *et al.*, 1983). This is a likely explanation because the NMDA receptor is known to be critically involved in the imprinting process in day 2 chicks (McCabe and Horn, 1988, 1991; McCabe *et al.*, 1992; Johnston *et al.*, 1993). However, the action of ketamine is not only limited to the NMDA receptor. Ketamine is a member of the family of PCP-like compounds, the pharmacology of which is complex (Iversen *et al.*, 1989, p. 218).

Two types of PCP receptors have been identified. One receptor, PCP site 1 is associated with the cation channel of the NMDA receptor, while the other, PCP site 2 is associated with the dopamine re-uptake carrier system (Rothman *et al.*, 1989). Additionally, ketamine has been shown to non-competitively reduce cholinergic responses (Ikemoto, 1986) and inhibit catecholamine uptake mechanisms. There is a possibility that the action of ketamine in extending the sensitive period is not mediated by the NMDA receptor but is due to an action on, or interaction with, other sites. Thus, in order to determine if the effect is indeed induced by an action on the NMDA receptor a more specific NMDA receptor antagonist was tested.

MK-801 is a potent and selective non-competitive NMDA receptor antagonist and acts at the cation channel of the NMDA receptor complex (Wong *et al.*, 1986). MK-801 is highly selective for PCP site 1, but unlike phencyclidine has little affinity for PCP site 2 (Reid *et al.*, 1990; Akunne *et al.*, 1991). In mammalian species it is some 60 times more potent as an antagonist of the NMDA receptor than ketamine (Martin and Lodge, 1988). An advantage of MK-801 over a competitive NMDA receptor antagonist such as AP5 is its lipophilic nature that allows it to easily enter the brain when systemically administered (Iversen *et al.*, 1989).

The present experiment investigated whether MK-801 administered 10 h post-hatching has the same effect on the ability of chicks to imprint on day 8 post-hatching as does ketamine, in order to provide more evidence as to the involvement of the NMDA receptor in this process.

6-3.1 Methods

MK-801 was a gift from Merck, Sharpe and Dohme Australia Pty. Ltd.

Thirty-four australorp × white leghorn chicks of either sex were used in this experiment. Incubation and rearing conditions were as described in Chapter 2 (see page 42). All handling prior to the imprinting procedures, including injecting the chicks, was done in complete darkness. Sixteen chicks received 5 mg/kg of MK-801 dissolved in 0.1 ml of sterile pyrogen-free 0.9% saline injected intramuscularly. Eighteen control chicks received 0.1 ml of the vehicle. Pilot experiments were performed to determine the appropriate dose of MK-801. The dose settled on was a compromise between producing a depth of anaesthesia similar to KX, without producing a prolonged period of hypertonus. Nevertheless, the dose of MK-801 that was used caused hypertonus in some of the chicks for a period of up to 6-8 h. Twelve hours after the injection the chicks were removed from the incubators and placed in the group-rearing cages. On day 8 the chicks were exposed to the imprinting stimulus for 2 h. As all of the previous experiments have indicated that the effect of extending the sensitive period is specific to those chicks trained on the hen, in this experiment only the hen was used as an imprinting stimulus.

6-3.2 Results

6-3.2.1 Activity in training

The mean ± SEM activity of the chicks during the training period is presented in Table 6-6. Activity, measured in revolutions of the imprinting wheels was log transformed and analysed using a two-factor analysis of variance with a repeated measure. The factors were treatment (saline or MK-801) and direction of movement (towards or away from the imprinting stimulus). There was no main effect of treatment ($F_{1,32} = 1.85$, $p = 0.18$) or direction of movement ($F_{1,32} = 0.97$, $p = 0.33$), and no interaction between the factors treatment and direction ($F_{1,32} = 0.61$, $p = 0.44$).

Table 6-6. Activity in training.

Treatment	Number trained	Total activity (revolutions)	Activity towards (revolutions)	Activity away (revolutions)
Saline	18	163 ± 48	87 ± 26	76 ± 22
MK-801	16	340 ± 127	221 ± 118	119 ± 40

Mean ± SEM number of revolutions of the imprinting wheels during the training period. The total activity as well as the activity towards and away from the imprinting stimulus is presented. There was no significant difference between the activity of the two groups.

6-3.3.2 Activity during the testing periods

The mean ± SEM number of revolutions made by each group in both tests is presented in Table 6-7. The data was log transformed and analysed using two-tailed t-tests. In both tests there was no significant difference in the total activity of the chicks (1 h test, $t = 0.52$, $p = 0.62$; 24 h test, $t = 0.80$, $p = 0.43$, both two-tailed t-tests).

Table 6-7. Activity in the preference tests.

Treatment	Test 1 hour after training		Test 24 hours after training	
	Reached activity criterion n %	Total activity (revolutions)	Reached activity criterion n %	Total activity (revolutions)
Saline	6 33	5 ± 3	14 78	5 ± 2
MK-801	6 38	7 ± 4	12 75	3 ± 1

Mean ± SEM activity of the chicks during the testing periods. Activity is measured in revolutions of the imprinting wheels. Also presented is the number (n) and the percentage of chicks that reached the activity criterion. There was no significant difference between the activity of the groups in either of the testing periods.

6-3.3.2.1 Number of chicks that reached the activity criterion

The proportion of chicks reaching the activity criterion is presented in Table 6-7 and was analysed using the weighted least squares approach that is described on page 55. Significantly fewer chicks reached the activity criterion in the test 1 h after training compared to the test 24 h after training ($X^2 = 14.23$, $df = 1$, $p < 0.005$). The number of

chicks reaching the activity criterion was not affected by treatment ($X^2 = 0.00$, df = 1, $p > 0.95$).

6-3.3.3 Percent preference scores

The mean percent preference scores of the groups are presented in Figure 6-4. The percent preference scores for both testing periods are presented on the same axis, although the results were analysed separately. The data was arcsine transformed and the difference between the groups was analysed using t-tests. T-tests were also used to determine if the percent preference scores differed significantly from the no-preference level.

In the test 1 h after training there was no difference between the mean percent preference scores of the two groups ($t = p = 0.58$, two-tailed t-test), and neither of the groups differed significantly from the no-preference level of 50% ($p > 0.05$, two-tailed t-test).

In the test 24 h after training the mean percent preference score of the MK-801 treated group was significantly greater than the mean percent preference score of the saline-treated group ($t = -2.50$, $p = 0.02$, two-tailed t-test). Also, the percent preference score of the MK-801 treated group was significantly greater than the no-preference level of 50% ($t = 3.04$, $p = 0.01$, two-tailed t-test).

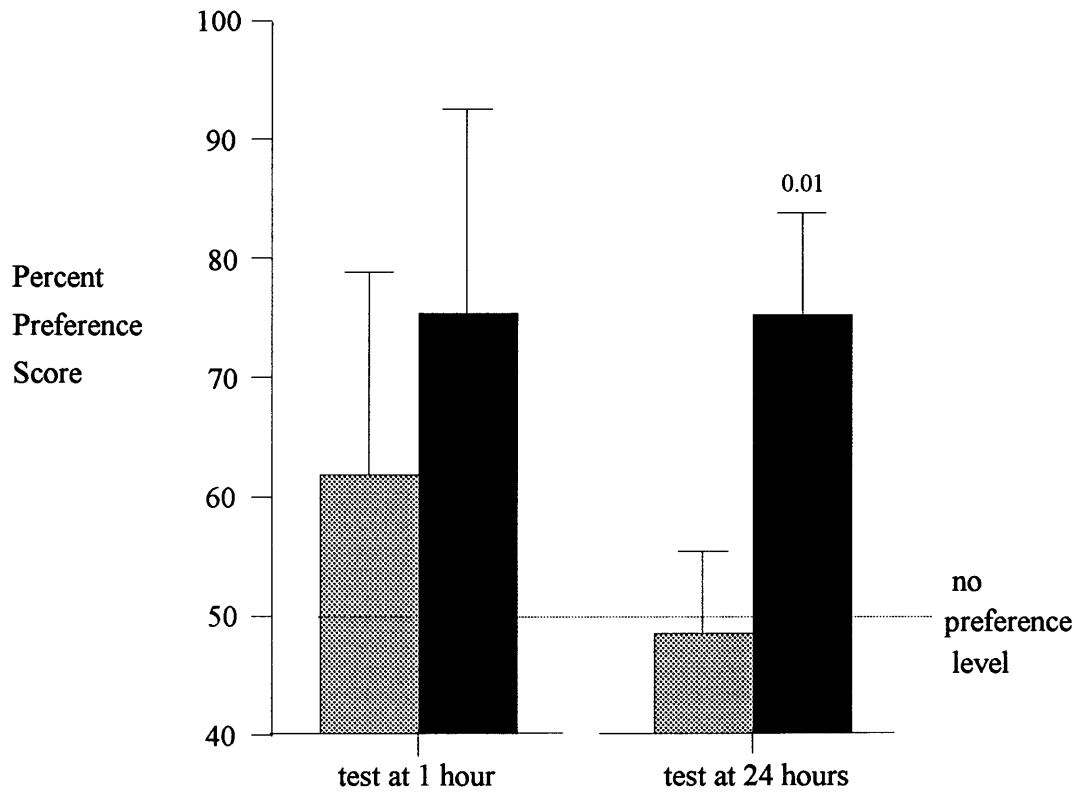


Figure 6-4. Percent preference scores of MK-801 and saline-treated chicks tested 1 h and 24 h after training.
Mean percent preference scores ± SEM are presented. Grey bars represent the saline-treated group, black bars represent the MK-801-treated group. Only the hen was used as an imprinting stimulus. The difference between the groups 1 h after training was not significant, nor were the preferences for the imprinting stimulus significant at this time. When tested 24 h after training, the preference of the MK-801 treated group was significantly higher than that of the saline-treated group, and the no-preference level of 50%. See text for p values.

6-3.3 Discussion

This experiment provides evidence that the previously shown (Chapters 4, 5 and Experiments 1 and 2 of this chapter) ability to display filial behaviour on day 8 in chicks treated with a mixture of KX or ketamine alone is mediated through the NMDA receptor. Compared to the saline-treated chicks, chicks treated with MK-801 10 hours after hatching and exposed to a hen on day 8 post-hatching showed a significantly greater

preference for the imprinting stimulus in the test given 24 h after training. The saline-treated chicks performed at the chance level, while the preference shown by the MK-801-treated group was significantly greater than the no-preference level of 50%. The specificity of MK-801 for the NMDA receptor (Iversen *et al.*, 1989) indicates that this receptor system is able to mediate the extension of the sensitive period.

The mean percent preference score of the MK-801 treated group in the test 1 h after training did not differ significantly from the no-preference level. This is despite the fact that the percent preference score of the MK-801-treated group at this time is practically the same as the percent preference score of the group in the test 24 h after training. This can be explained by the lower number of animals reaching the activity criterion and the higher variability of the group at this time, evidenced by the higher SEM. A lower number of animals reaching the activity criterion in the test 1 h after training has been a consistent characteristic of the imprinting behaviour of chicks at this age.

The saline-treated group did not show a significant preference for the imprinting stimulus in either of the imprinting tests. This group did not differ significantly from the MK-801 treated group in the test 1 h after training, but showed significantly less preference for the imprinting stimulus than the MK-801-treated group in the test 24 h after training.

In all parameters of filial behaviour that have been measured in this experiment, the results obtained through treatment with KX have been mimicked by MK-801, which is known to be a more potent antagonist of the NMDA receptor than ketamine. The results strongly suggest that the sensitive period for the formation of a filial preference on the hen is extended through modulation of the NMDA receptor.

6.5 Chapter Discussion

This chapter has demonstrated that the effect of extending the sensitive period which was shown in previous chapters through KX treatment is mediated primarily by the NMDA receptor. Evidence for an involvement of the NMDA receptor in the extended

sensitive period was obtained in Experiment 6-2 in which the duration of action of ketamine by itself was matched with the duration of action of KX. Under these circumstances ketamine treated chicks showed an extended sensitive period, while the group of chicks treated with xylazine for a comparable time did not show imprinting on day 8. Experiment 6-3 demonstrated that a more potent non-competitive NMDA receptor antagonist was also capable of extending the sensitive period for imprinting.

It should be noted that in every experiment of this chapter it was only the hen-trained groups that were able to imprint. This supports the findings of the previous chapter in which only those groups that were treated with KX at 10, 20 or 40 h post-hatching and which were trained on the hen showed a significant preference for the training stimulus in the test 24 h after training. In the previous chapter it was suggested that the predisposition mechanism had been affected by the treatment, and this possibility will be explored in the following chapter.

A specific role of the NMDA receptor in the extended sensitive period was indicated by the results of Chapter 4 in which [^{3}H]-MK-801 binding was significantly increased in the left IMHV of KX-treated chicks. This provides evidence that the NMDA receptor is involved in imprinting on day 8, and, coupled with the results from the present chapter in which antagonism of the NMDA receptor has been shown to be important in extending the sensitive period, it is probable that the mechanism underlying this extension is through a modulation of NMDA receptors. Chapter 4 also showed that before imprinting, NMDA receptor density was suppressed in KX-treated chicks, compared to saline-treated chicks, but increased after exposure to the imprinting stimulus. Therefore, the effect of the treatments may be to preserve the ability to form an NMDA receptor mediated imprinting memory.

McCabe and Horn (1988, 1991) have proposed that the increased number of NMDA receptors in the left IMHV 8 h after training is related to the formation of a long-term memory store. They (McCabe and Horn, 1991) have demonstrated that changes in NMDA receptor binding in the left IMHV do not occur until 6-8½ h after training. It follows that the preferences of the chicks 1 h after training may not be a reflection of a

long term (NMDA receptor mediated) memory, but may instead represent an intermediate form of memory. When tested 24 h after training, memory may be dependent upon NMDA receptor mediated events.

Thus, the extended sensitive period may be due to a modulation of NMDA receptors which allows a long-term imprinting memory to be formed. A key point in the modulation of NMDA receptors is probably the fact that the treatment must be given at a very sensitive period in the development of the chick. In Chapter 5 it was shown that the treatment was ineffective if it was given outside of the sensitive period for imprinting, suggesting that it needed to act on the neural systems underlying imprinting before they had been modified by maturational or experiential changes.

To reiterate a key point made in Chapter 4 and supported by the results of Chapter 5, the treatments that have been shown to extend the sensitive period (KX at 10, 20 or 40 h or 2 doses of ketamine alone or MK-801) probably do so by modulating the expression of a subtype of NMDA receptor that is favourable in the formation of an imprinting memory. Carmignoto and Vicini (1992) have shown that the developmental change in duration of the NMDA stimulated excitatory post-synaptic current (attributed to a change in NMDA receptor subunit composition) is delayed by dark-rearing or infusion of tetrodotoxin into the visual cortex. Both of these procedures decrease the activity of the visual cortex, and are parallelled in the present experiment by antagonism of the NMDA receptor and dark-rearing. After treatment, chicks should still be able to imprint, but the dark environment might prevent them from doing so to a satisfactory degree. When subsequently exposed to a stimulus that is more attractive than the dark-environment they may form an imprinting memory of that stimulus. By allowing the chicks to form a stronger representation of their environment prior to imprinting it may be more difficult for them to form an imprinting memory of one of the test stimuli on day 8 post-hatching. The experiment that is reported in Chapter 8 investigated the ability of light-reared, isolated chicks to imprint on day 8.

Given that the treatment is only effective before the sensitive period for imprinting ends, the possibility that the treatments (KX, 2 doses of ketamine or MK-801) prevent an

imprinting memory from forming must be considered. In the experiments of this thesis the inability of untreated chicks to imprint has been attributed to their having already imprinted, either on the dark environment or on some other stimulus, perhaps of an auditory nature. Antagonists of the NMDA receptor are capable of preventing imprinting (McCabe *et al.*, 1992), but it is doubtful that the treatments given in this thesis have the ability to prevent an imprinting memory from forming for the whole period of dark-rearing, especially when considering that the immediate behavioural effects of MK-801 (the strongest of the treatments) are absent after 12 h. This raises the question of whether the treated chicks have imprinted during the period of dark-rearing, but have the ability to continue to imprint on other stimuli. Viewed another way, the treated chicks may not have the ability to close the sensitive period, or may form a secondary attachment to a stimulus if it is sufficiently attractive. This provides an alternative explanation for the fact that a filial attachment occurs on the hen only.

The fact that the single dose of ketamine was ineffective in producing an extended sensitive period when it was administered without xylazine suggests that a synergistic effect between the two drugs occurs to produce the effect. Xylazine inhibits the breakdown of ketamine in the liver, which may prolong the action of ketamine (Kharasch *et al.*, 1992). When ketamine is injected intramuscularly, it is absorbed into the blood and diffuses into the central nervous system. By inhibiting its breakdown its concentration may be maintained in the circulation, thereby maintaining its concentration in the central nervous system for a longer period of time than if ketamine were given by itself.

It is possible, however, that the effect of extending the sensitive period is due to a combined action of the drugs on the systems that control imprinting behaviour. Through actions on NMDA and non-NMDA receptors, excitatory amino acid agonists have been shown to stimulate the release of noradrenaline from noradrenergic axonal terminals in rat hippocampus (Pittaluga and Raiteri, 1992) and rat hypothalamus (Blandina *et al.*, 1992). These effects are antagonised by a number of NMDA receptor antagonists including MK-801. MK-801 and other PCP-like compounds can also inhibit the

noradrenaline uptake carrier in the rat (Massamiri and Duckles, 1991). Although it is not certain that these interactions between the NMDA receptor and the noradrenergic system exist in the chick brain, the result of Experiment 1 of this chapter suggest that they may occur. Thus, it is possible that both systems are involved in the extension of the sensitive period for imprinting. It is even possible that ketamine mediates its effect through modulating the noradrenergic system.

6.6 Conclusion

Treatment with a single dose of ketamine (55 mg/kg) failed to produce an extension of the sensitive period for imprinting. Likewise, a single dose of xylazine (6 mg/kg) was ineffective in producing the action. However, when the action of ketamine was prolonged by injecting it twice (at 10 h and 12 h post-hatching) the chicks were able to imprint on day 8. The same was not true for xylazine treatment. This indicates that an action on the NMDA receptor system is responsible for the effect shown, further supported by the fact that chicks treated with MK-801 10 h after hatching also imprinted.

There is obviously some synergism between ketamine and xylazine because together, in single doses, they extend the sensitive period for imprinting. It is suggested that the synergy between the two drugs is related to the altered biotransformation of ketamine through the actions of xylazine. Effectively, this may produce an increase in the amount of ketamine that is circulating and may account for the greater anaesthesia produced when the drugs are given together. The anaesthetic effect itself is not thought to be directly related to the extension of the sensitive period because chicks treated with the double dose of xylazine were anaesthetised for a comparable amount of time, yet showed no filial behaviour on day 8.

Chapter 7

Does Ketamine-Xylazine Treatment Induce a Predisposition?

7.1 Introduction

In the previous two chapters the demonstration of filial behaviour in day 8-9 chicks has been shown in the groups of chicks that were trained on the hen (but not the box) and treated with either the mixture of KX, two doses of ketamine alone, or MK-801. The specificity of the effects of these treatments on the groups of chicks that were trained on the hen raises the possibility that it is not an imprinting *learning* process that is occurring, but rather the preference for the hen may be due to the development of a predisposition to approach a conspecific, as has been shown to occur in chicks aged 24 and 36 h post-hatching (Johnson *et al.*, 1989).

In chicks aged 24 or 36 h post-hatching a predisposition to approach a stuffed jungle fowl occurs as a result of, ostensibly, non-specific stimulation normally associated with the training process of imprinting. The predisposition has been shown to develop after training chicks in an operant learning procedure (Johnson *et al.*, 1985) and by placing chicks in imprinting wheels with or without an imprinting stimulus being present (Bolhuis *et al.*, 1985). However, chicks that received only half an hour of light exposure during a priming period, but were otherwise left in the dark incubator did not develop the predisposition (Johnson *et al.*, 1985). The predisposition is not immediately evident, but is manifest 24 h after training. It has thus been termed a developing predisposition (Johnson *et al.*, 1985).

One of the only constraints on the development of the predisposition is that the chick must receive the non-specific stimulation during a specific period of development.

Johnson *et al.* (1989) reported that a predisposition developed in chicks that had been placed in imprinting wheels at 24 and 36 h post-hatching but not at 12 or 42 h post-hatching. There is thus a sensitive period for the development of the predisposition, which is well outside the time-frame of the present experiment. Nevertheless, in the experiments of this thesis, the chicks that did display a preference for the hen on day 9 might have had a delayed or extended sensitive period for the development of the predisposition, as has been shown to occur in DSP4 treated chicks (Davies *et al.*, 1992).

Superficially at least, the results presented in this thesis, in which a preference for an imprinting stimulus has been shown only by those chicks that have been trained on the hen, are compatible with a predisposition having developed. The fact that control chicks did not show a preference for the hen, but that chicks that were treated with KX, two doses of ketamine or MK-801 did show a preference, raises the possibility that the sensitive period for the development of the predisposition may have been extended. The present experiment seeks to determine if a predisposition for the hen can, in fact, be induced in chicks treated with KX.

7.2 Methods

7.2.1 Subjects

Thirty chicks were used in this experiment, 16 chicks were treated with the same dose of KX that was used in the previous experiment (55 mg/kg ketamine and 6 mg/kg xylazine made up to a volume of 0.1 ml with 0.9% sterile pyrogen free saline) and 14 chicks were treated with the vehicle (saline). Both treatments were administered 10 h after hatching.

7.2.2 Experimental procedure

The chicks in this experiment received exactly the same experimental treatment as the chicks in the previous imprinting experiments, except that during the training period no stimulus was present. Groups of five chicks were removed from the dark-room in a

light-proof box. Each chick was primed according to the method on page 51. After the priming period the chicks were placed in the imprinting wheels for a 2 h period. While they were in the imprinting wheels, the spot-light that normally illuminated the imprinting stimulus was turned on. These chicks thus received a similar amount of light exposure as the chicks in the previous experiments (chapters 3 through 7). In chicks aged 24 or 36 h, the predisposition can be induced with or without a light source during the period in the wheels (Bolhuis *et al.*, 1985; Johnson *et al.*, 1985). Therefore, there is no reason to suspect that the presence or absence of light should make a difference to the development of the predisposition, at least in 1-2 day old chicks. However, in day 8 chicks, light might play an important role, even if it only serves to provide a longer period of visual priming. After the period in the wheels the chicks were returned to the dark-rearing room. They were removed from this room for testing 1 h and 24 h after the experience in the imprinting wheels. The chicks were tested using a simultaneous choice test, as has been used in the previous experiments (see page 51).

7.2.3 Statistical analysis

Comparisons between the KX-treated group and the saline-treated group for all measures (total activity during the 2 h period spent in the wheels, total activity in both testing periods and percent preference scores) were analysed using t-tests. In order to determine if a predisposition for the hen was present, the activity of the chicks towards the hen was expressed as a percentage of the total activity (see page 54). T-tests comparing the percent preference scores with the no-preference level of 50% were also performed. The activity data was log transformed before analysis, and an arcsine transformation was applied to the percent preference scores (Winer, 1971).

7.3 Results

7.3.1 Activity while in the wheels

The activity while in the imprinting wheels for 2 h, measured in revolutions of the imprinting wheels, is presented in Table 7-1. There was no significant difference between the saline-treated group and the KX-treated group ($t = -1.05$, $p = 0.30$, two-tailed t-test).

Table 7-1. Number of chicks in each group and their activity during the 2 hour period spent inside the wheels.

Treatment	Number trained	Total activity in training (revolutions)
Saline	14	619 ± 109
KX	16	787 ± 117

Mean ± SEM total activity during the 2 h period spent in the imprinting wheels. There was no significant difference between the activity of the chicks. Also presented is the number of chicks in each group.

7.3.2 Activity in the preference tests

The total activity of the two groups in each of the preference tests and the percent of chicks reaching the activity criterion in each test is presented in Table 7-2. The data was log transformed and analysed separately for each testing period. There was no significant difference between the activity of the two groups of chicks in the test 1 h after experience in the imprinting wheels ($t = -0.25$, $p = 0.80$, two-tailed t-test) or in the test 24 h after experience in the imprinting wheels ($t = -0.61$, $p = 0.55$, two-tailed t-test).

Table 7-2. Activity in preference tests

Treatment	1 hour after experience in the imprinting wheels			24 hours after experience in the imprinting wheels		
	Reached activity criterion		Total activity (revolutions)	Reached activity criterion		Total activity (revolutions)
	n	%		n	%	
Saline	11	79	4.6 ± 2	11	79	6.6 ± 2
KX	7	44	8.4 ± 5	12	75	8.3 ± 4

Mean ± SEM total activity during both testing periods. Activity is presented in revolutions of the imprinting wheels. There were no significant differences in the activity of the groups in either of the tests. Also presented in the table is the number (n) of chicks in each group that reached the activity criterion in each of the testing periods.

7.3.2.1 Number of chicks that reached the activity criterion

The proportion of chicks reaching the activity criterion was analysed using the weighted least squares approach that is described on page 55. There was no difference between the groups in the number of chicks that reached the activity criterion ($X^2 = 2.57$, df = 1, $p > 0.10$), nor was there an effect of the time that the chicks were tested ($X^2 = 1.69$, df = 1, $p > 0.10$).

7.3.3 Percent preference scores

The activity of the chicks towards the hen is expressed as a percentage of the total activity in each preference test. These scores are presented in Figure 7-1. The scores in each of the testing periods were analysed separately. There was no significant difference between the groups in their percent preference scores in the test 1 h after experience in the imprinting wheels ($t = 1.58$, $p = 0.14$, two-tailed t-test) or in the test 24 h after experience in the imprinting wheels ($t = -0.13$, $p = 0.90$, two-tailed t-test). The group treated with KX did show a significant preference for the hen in the test 1 h after experience in the imprinting wheels ($t = 3.25$, $p = 0.02$, two-tailed t-test). However, their percent preference score in the test 24 h after experience in the imprinting wheels

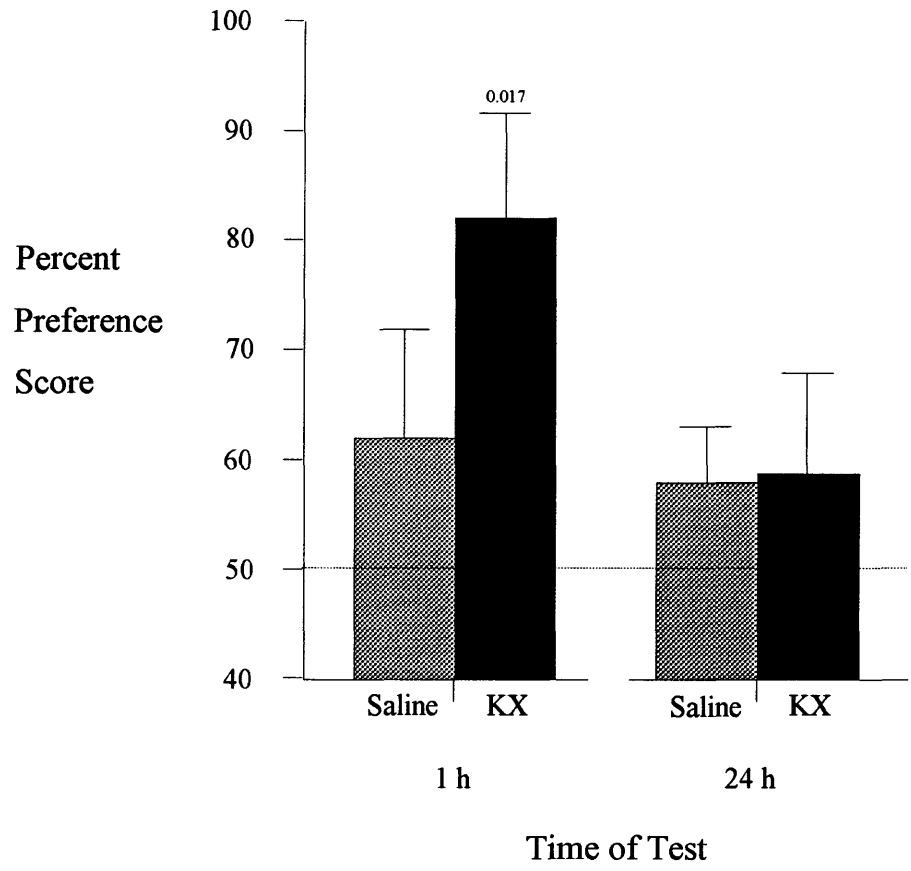


Figure 7-1. Mean ± SEM percent preference scores of untrained chicks treated with KX or saline. Activity towards the hen is expressed as a percentage of the total activity in the testing period. Percent preference scores from the test 1 h and 24 h after experience in the imprinting wheels are presented. There was no significant difference between the groups in the test 1 h or 24 h after experience in the imprinting wheels. Only the group treated with KX showed a significant preference for the hen, although this was only in the test 1 h after experience in the imprinting wheels ($p = 0.017$). The dashed line shows the no-preference level of 50%.

was not significantly different to the no-preference level of 50% ($t = 1.05$, $p = 0.32$, two-tailed t-test). The saline treated chicks showed no significant departure from the no-preference level of 50% in the test 1 h after experience in the imprinting wheels ($t = 1.14$, $p = 0.28$. two-tailed t-test) or in the test 24 h after experience in the imprinting wheels ($t = 1.47$, $p = 0.17$, two-tailed t-test).

7.3 Discussion

It has been suggested (see page 99) that the preference shown for the hen 24 h after training in chicks treated with KX, two doses of ketamine or MK-801 might be due to the development of a predisposition to approach a hen and not due to a learning effect. In the present experiment a predisposition was not shown in the test 24 h after the experience in the imprinting wheels. The percent preference scores of both groups did not differ significantly from the no-preference level of 50%. If a predisposition, similar to that which occurs in chicks 24 h old was to have developed, it should have been evident at this time.

In the test 1 h after the experience in the imprinting wheels, a significant preference for the hen was displayed by the KX-treated group, but not by the group treated with saline. In this test, less than half of the chicks reached the activity criterion in the KX-treated group, compared to 75% of the saline-treated chicks. Nevertheless, the hen was the preferred stimulus by the KX-treated chicks. This did not, however, extend to the test 24 h after the experience in the imprinting wheels. This is in contrast to the situation that occurs when chicks are placed in the wheels at 24 or 36 h post-hatching (Johnson *et al.*, 1989). In younger chicks, the predisposition does not become evident until 24 h after experience in the imprinting wheels.

The relationship between the preference for the hen, shown by the KX-treated chicks in the test 1 h after experience in the imprinting wheels and the predisposition in younger chicks, which only becomes evident 24 h after experience in the imprinting wheels, is unclear (Johnson *et al.*, 1985). One does not wish to discount the preferences of the KX-treated chicks in the test 1 h after experience in the imprinting wheels, but this is clearly not characteristic of that which would normally be expected to occur in younger chicks that exhibit a predisposition. Furthermore, throughout the experiments of this thesis, it has become apparent that the preference shown by the chicks 1 h after training is not necessarily indicative of the preferences in the test 24 h after training. Indeed, this

instability of preferences is one of the reasons why the experiment investigating the occurrence of predispositions was conducted, as clear differences between the groups emerged only in the test 24 h after training. In this experiment too, the preferences shown in the test 1 h after experience in the imprinting wheels do not provide a good indication of the preferences in the test 24 h after experience in the imprinting wheels.

These results are significant to the thesis as a whole because they demonstrate that in day 8 chicks treated with KX or saline, a predisposition to approach a conspecific does not develop as it does in chicks 24-36 h old. In the experiments in which day-8-trained chicks showed a preference for a hen, it can confidently be stated that this is due to learning having occurred, and not due to the development of a predisposition. In other words, the preference for the hen would not have become evident unless the chicks had been exposed to the hen, and thus learning must have occurred. Therefore, the significant preferences shown for the hen in previous experiments can be attributed to the classical type of imprinting and not due to a developing predisposition.

An alternative explanation for the fact that imprinting only occurred on the hen is that the hen provides a more complex visual stimulus than the box and, is thus more attractive as an imprinting stimulus to day 8 chicks. Berryman *et al.* (1971) reported that 5 and 6 day old chicks preferred a stimulus that was more visually complex, while day 2 chicks showed no preference between a complex stimulus and a more simple one. The stimuli used by Berryman *et al.* (1971) were 2-dimensional sheets of cardboard with different grid-patterns on them. The hen used in the present experiment is of much greater complexity and may thus be even more appealing to the chicks. Additionally, features such as the head and neck region have been shown to be particularly important in the elicitation of filial responses, albeit for predispositions (Johnson and Horn, 1988). Nevertheless, these features might contribute to the effectiveness of the hen as an imprinting stimulus.

Bateson (1991, p. 120) proposes that feature detectors are sensitive to discrete units including lines, colours and head shapes. If a chick possesses a mechanism whereby it can identify stimuli such as the head and neck region, and respond to these features in a

filial manner, it follows that this may increase the arousal of an animal, thus increasing its ability to learn. Through a mechanism similar to this, it is not unrealistic to propose that some features of the hen may increase its effectiveness as an imprinting stimulus, but this does not necessarily infer that an unlearnt predisposition is present.

Boakes and Panter (1985) have shown that the degree to which secondary imprinting occurs is dependent on the strength of the primary imprinting stimulus. Chicks that had imprinted on a live hen were subsequently unable to imprint on a cup. In contrast, chicks that had been imprinted on a toy windmill, and then exposed to the cup, did show a preference for the cup. However, when tested on day 11, five days after the initial test, they no longer showed any effects due to exposure to the cup. In terms of ranking the imprinting stimuli, the live hen was the most effective stimulus, followed by the moving windmill and then the cup. It is not suggested that the stuffed hen used in the present experiment is as effective as the live hen used by Boakes and Panter (1985), which was able to vocalise and was encouraged to move about its compartment and feed during the training periods, all of which probably enhanced its value as an imprinting stimulus. However, this does illustrate that some stimuli are more effective than others in eliciting imprinting behaviours, which could account for the fact that treated chicks (with KX at 10, 20 or 40 h after hatching or MK-801, or the double dose of ketamine) that were trained on the hen were able to demonstrate imprinting behaviour 24 h after training.

In the test 1 h after experience in the imprinting wheels, in which a significant preference for the hen was shown by the KX treated chicks, it did appear as though the hen was a more effective imprinting stimulus. Furthermore, in the test 24 h after experience in the imprinting wheels, in both groups, the slight preference that was present was towards the hen. While this was non-significant, the consistent tendency for both groups to approach the hen, supports the idea that, at this age, the hen is a more attractive imprinting stimulus than the box. Interestingly, the results presented in Chapter 3 in which chicks were trained on days 2, 4, or 6 suggest the opposite (see Figure 3-1 on page 63). In that experiment it appeared as though the box was the more attractive imprinting stimulus on days 2 and 4. Coupled with the results of the day 8

chicks it appears that there is a change in the preference of the chicks with age. Younger chicks seem to prefer a less complex stimulus such as the box, and older chicks seem to prefer a more complex stimulus such as the hen.

7.4 Conclusion

This experiment has demonstrated that there is no predisposition for chicks treated with KX at 10 h post-hatching to approach a hen in preference to a box on day 9 post-hatching. The development of a predisposition had been proposed as an explanation for the fact that the extended sensitive period was only evident in hen-trained chicks. Instead, it is suggested that some property of the hen makes it a more effective imprinting stimulus on day 8 post-hatching.