

**PRENATAL APPETITIVE LEARNING
ABOUT ETHANOL IN THE RAT:
POSSIBLE REINFORCERS**

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*Nere ama eta izebari,
Izandako pazientiagatik eta baldintzarik
gabeko maitasun guztiagatik.*

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*"The important thing is not to stop questioning.
Curiosity has its own reason for existing. Never
lose a holy curiosity."*

Albert Einstein (1879 - 1955)

ABSTRACT

Rat fetuses can perceive and learn about chemosensory stimuli derived from their mother's diet. Previous studies conducted in this laboratory have shown that prenatal exposure to ethanol during the final days of gestation increases the acceptance and liking of an ethanol flavor in infant and adolescent rats. Similar results, however, were not found after prenatal exposure to other non-ethanol flavors. The enhanced acceptance of ethanol has been shown to be an appetitive response acquired prenatally, and mediated by the opioid system. The main objective of this thesis was to investigate further the identity of the reinforcer that may be acting during ethanol exposure on the final days of gestation, and by which rat fetuses seem to learn a conditioned preference for this flavor. The identity of two possible reinforcers that may be activating the opioid system was considered: the amniotic fluid (with its KIF component) and the pharmacological effects of ethanol. In addition, we investigated the role played by the first metabolite of ethanol, acetaldehyde, on the appetitive response acquired by ethanol following its prenatal exposure. The results of all the experiments indicate that prenatal experience with a non-ethanol flavor without pharmacological effects may result in an increased attraction for this stimulus, detected soon after birth. This might be taken as evidence to support the notion that the KIF component of the amniotic fluid serves as the

reinforcer in a prenatally acquired appetitive response. However, this reinforcer appears to be rather weak, since the learned response is not retained for a relatively long period of time, even when using techniques that are more suitable for measuring behavior at this developmental stage. It is also concluded that the activation of the mu-opioid receptor system appears to be critical, not only for prenatal appetitive learning about ethanol, but also about other non-ethanol flavors. While kappa-opioid receptors appear to participate only in the prenatally learned response to ethanol. Finally, the results suggest that acetaldehyde, rather than ethanol, appears to be the main reinforcer involved in prenatal appetitive learning about the flavor of ethanol.

LABURPENA

Jakina da arratoi fetuek amagandik jasotako estimulu kimio-sentsorialak hautematen dituztela eta haiei buruz ikasten dutela. Gure laborategian eginiko ikerketek frogatudute etanola ernaldiaren azken egunetan administratuz gero droga honen kontsumoa handitzen dela bai arratoi kumeetan eta baita arratoi nerabeetan ere. Efektu hau, ordea, ez da inoiz aurkitu efektu farmakologikoa ez duten usain edo zaporeak erabiliz. Gainera, etanolaren kasuan, ernaldiaren esposizioaren ondorioz ematen den kontsumo handitu horretan sistema opioideak parte hartzen duela frogatu da. Tesi honen helburu orokorra jaio-aurreko etapan etanolaren esposizioak sortzen duen ikaskuntza appetitiboan (gurarian) parte hartzen duten indartzaileen inguruan ikertzea izan da. Sistema opioidea aktibatzen duten bi indartzaile hartu ditugu kontutan: likido amniotikoa (eta bertako RIF osagaia) eta etanolaren efektu farmakologikoa. Horretaz gain, etanolaren lehen metabolitoak, azetaldehidoak, ernaldian zehar garatutako etanolarekiko erantzun appetitibo horretan duen erantzukizuna aztertu da. Esperimentuen emaitzek adierazi zuten efektu farmakologiko gabeko estimuluen jaio-aurreko esposizioak ere jaio ondoren estimulu horienganako erakarpena handitzen zutela, erantzuna jaio bezain azkar neurtu ezker. Emaitza hau RIF-en paperaren aldeko ebidentzia bat litzateke, baina indar handirik gabeko indartzailea da, izan ere jaio-aurreko ikaskuntza horrek ez du

gehiagi irauten arratoi kumeetan. Horrez gain, emaitzek adierazten dute sistema opioidearen barnean, mu errezeptoreen papera kritikoa dela, ez bakarrik etanolaren inguruan ikasteko, baizik eta efektu farmakologikorik gabeko sustantzien inguruan ikasteko ere. Bestalde, ikusten da kappa errezeptoreek etanolaren kasuan bakarrik parte hartzen dutela. Azkenik, badirudi, etanolaren inguruko jaio-aurreko ikaskuntza honetan etanolak berak baino aztealdehidoak paper garrantzitsuago bat jokatzen duela. Datu guzti hauek kontutan hartuta, esan dezakegu azetaldehidoak jaio-aurreko etanolarekiko ikaskuntzan jokatzen duen papera oso garrantzitsua dela, eta gainera posible dela metabolito hori izatea mu eta kappa errezeptore opioideak aktibatzen dituena.

RESUMEN 1

Los fetos de rata perciben estímulos quimio-sensoriales provenientes de la dieta materna y aprenden sobre ellos. Estudios previos de nuestro laboratorio han demostrado que la exposición prenatal al etanol durante los últimos días de la gestación aumenta la aceptación del sabor del etanol, tanto en ratas infantiles como en adolescentes. Sin embargo, con la exposición prenatal a sabores no-etílicos no se encontró este efecto a esas edades. Este aumento en la aceptación del etanol se ha demostrado que es una respuesta condicionada adquirida prenatalmente y que está mediada por el sistema opioide. El objetivo general de esta tesis es investigar la identidad del reforzador que puede estar actuando durante la exposición prenatal al etanol y por el cual el feto de rata adquiere una respuesta apetitiva hacia el sabor del etanol. Dos posibles reforzadores que activan el sistema opioide fueron considerados: el líquido amniótico (y su componente KIF) y los efectos farmacológicos del etanol. Además, también investigamos el rol que juega el primer metabolito del etanol, el acetaldehído, en la respuesta apetitiva adquirida después de la exposición prenatal a esta sustancia. Los resultados de todos los experimentos indican que la experiencia prenatal con sustancias no-etílicas y sin efectos farmacológicos produce un incremento en la atracción por las mismas cuando se evalúan en la etapa neonatal. Esto podría ser una evidencia de que el componente KIF del líquido amniótico funciona

como reforzador para la respuesta apetitiva adquirida en utero. Sin embargo, este parece ser un reforzador débil ya que el aprendizaje prenatal no es retenido durante un periodo largo de tiempo, aún utilizando técnicas de evaluación apropiadas para esa etapa del desarrollo. También se concluye que la activación del sistema de receptores opioides mu es crítico, no solo para el aprendizaje apetitivo prenatal sobre el etanol, sino también sobre sustancias no-etílicas. En cambio, el sistema opioide kappa parece estar mediando solamente la respuesta aprendida sobre el etanol. Por último, los resultados sugieren que el acetaldehído, más que el etanol, parece ser el principal reforzador del aprendizaje prenatal apetitivo sobre el sabor del etanol.

RESUMEN 2

Diversos estudios han demostrado que la exposición del feto a sabores y olores de la dieta materna afecta la respuesta del neonato a estos mismos sabores, generalmente produciendo un incremento del consumo o mostrando una preferencia en comparación con otros sabores n6veles. Este efecto se ha encontrado en diferentes especies animales: cerdos, perros, gatos, roedores y tambi6n en humanos. En ratas, por ejemplo, la exposici6n prenatal a diferentes sustancias como la manzana, el ajo y el citral, afectaron la respuesta postnatal a estas mismas sustancias elevando su consumo. Tambi6n se ha encontrado este mismo efecto con el etanol, una sustancia con caracteristicas quimiosensoriales y tambi6n efectos farmacol6gicos, que alcanza r6pidamente el l6quido amni6tico despu6s del consumo materno. Seg6n algunos autores este efecto o aprendizaje que produce la experiencia prenatal con sabores y olores se debe a la presencia de un reforzador que esta presente en el l6quido amni6tico de los 6ltimos d6as de gesti6n, denominado Kappa Impact Factor (KIF), que activa los receptores kappa del sistema opioide facilitando el aprendizaje apetitivo (Robinson & Menendez-Gallardo, 2010).

Estudios realizados en este laboratorio han encontrado en multiples ocasiones que la exposici6n al etanol en los 6ltimos d6as de gesti6n de la rata incrementa el consumo de

etanol y la palatabilidad de su sabor, cuando se miden al día postnatal 14 (infancia de la rata). Además, se ha demostrado que el efecto está mediado por el sistema opioide, y en especial por los receptores μ y κ , ya que su antagonización inhibe la observación de la respuesta apetitiva. Por el contrario, cuando las sustancias expuestas durante la gestación no tienen efecto farmacológico, no se encontró ningún aumento en la aceptación al sabor al día postnatal 14. De esta diferencia entre sustancias sin efecto farmacológico y el etanol se dedujo que los efectos farmacológicos del etanol podrían jugar un papel crucial en este aprendizaje prenatal. Sin embargo, no se puede descartar que la respuesta apetitiva hacia sabores no-etílicos se pueda detectar si la evaluación se realiza más cerca del nacimiento, en la etapa neonatal.

El objetivo general de esta tesis ha sido investigar la identidad del reforzador que actúa durante la exposición prenatal al etanol los últimos días de gestación y por el cual los fetos de rata adquieren el aprendizaje condicionado apetitivo sobre el sabor etílico. Más concretamente, el primer objetivo de la tesis fue observar si después de la administración prenatal a una sustancia sin ningún efecto farmacológico se encontraba una mayor aceptación de la misma realizando un primer test en el día postnatal 5 (que servía a su vez como recordatorio) y otro en el día 14. En un primer experimento se midió el consumo de etanol pero no se encontró ninguna respuesta diferencial. Sin embargo, en los siguientes dos experimentos, utilizando medidas más sensibles, se encontró

que la exposición prenatal a la vainilla producía una mayor respuesta operante hacia la vainilla (día postnatal 5) y una atracción por a ese mismo olor (día postnatal 1). En este último experimento también se encontró una mayor atracción neonatal por el etanol en sujetos expuestos prenatalmente a la droga. Estos resultados apoyaron la tesis de los autores previamente citados sobre el líquido amniótico y su agente KIF como reforzador.

El segundo objetivo de la tesis fué probar la implicación de los receptores mu y kappa en el aprendizaje prenatal apetitivo observado en los experimentos anteriores y de esta manera, probar la hipótesis del agente KIF como reforzador. En primer lugar se llevó a cabo un experimento con exposición prenatal a la sustancia no etílica, la vainilla, junto con antagonistas de los receptores opioides kappa o mu. El día después al nacimiento se midió la atracción por el olor a la vainilla. Sorprendentemente, antagonizar kappa no produjo ningún tipo de efecto ya que este grupo siguió mostrando una clara atracción por el olor a la vainilla. En cambio, el grupo administrado con el antagonista mu no mostró ninguna atracción por el mismo olor, demostrando así que el receptor clave para el aprendizaje apetitivo prenatal en este caso era mu, y no kappa. Por otro lado, en el siguiente experimento se siguió el mismo diseño, con la diferencia de que se administró etanol en lugar de vainilla. Los resultados, en este caso, demostraron que el antagonista mu inhibía completamente el aumento de la atracción al olor del etanol, y que antagonizar

kappa reducía (aunque no completamente) esta atracción confirmando los resultados obtenidos previamente. En conjunto, estos experimentos mostraron que es posible la detección del aprendizaje prenatal sobre los sabores y olores no etílicos siempre y cuando la evaluación se realice entre el nacimiento y el día postnatal 5. Además, se corroboró la importancia del sistema opioide en el aprendizaje de ambas sustancias: los receptores μ son cruciales en ambos casos, mientras que los receptores kappa solo están implicados en el aprendizaje sobre el etanol.

Por último, el tercer objetivo de la tesis fue comprobar la implicación del acetaldehído, primer metabolito en la cadena de oxidación del etanol, en el efecto observado después de la exposición prenatal al etanol. Estudios previos subrayan que este metabolito es el responsable de los efectos tanto aversivos como apetitivos que produce el etanol y que el etanol podría ser una "prodroga". Más concretamente, algunos estudios argumentan que el acetaldehído producido a nivel periférico en el hígado es el responsable de los efectos aversivos del etanol, y por el contrario, que el acetaldehído producido en el cerebro mediante las catalasas es el reforzador apetitivo. La importancia del acetaldehído ha sido probada en ratas adultas e infantes administrando un fármaco que secuestra el acetaldehído, anulando así los efectos reforzantes del etanol. Para comprobar la implicación del acetaldehído en el aprendizaje fetal sobre etanol, realizamos tres experimentos evaluando la respuesta en diferentes días

postnatales. En los tres casos, las ratas preñadas fueron administradas con etanol y el agente secuestrante (D-Penicillamina). Los resultados de los tres experimentos muestran que la eliminación del acetaldehído durante la exposición prenatal inhibe el aprendizaje apetitivo sobre el etanol, ya que los sujetos no mostraron aumento en la atracción por el olor, ni en las respuestas operantes hacia el etanol, ni tampoco en el consumo de esa sustancia. Estos datos sugieren que el rol que juega el acetaldehído en el aprendizaje prenatal apetitivo sobre el etanol es crucial, y probablemente sea este el que media la activación de los receptores opioides μ y κ .

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INTRODUCTION



This dissertation contains the work of a 4-year PhD project financed by a Basque Government pre-doctoral fellowship and directed by Dr. M Gabriela Chotro. Within this research area of prenatal exposure to alcohol in rats, which she has been leading since she arrived at the Department of Psychology in UPV/EHU, this thesis focuses on learning more about the reinforcing effects or mechanisms that take place while the fetus is inside the uterus and the mother is given alcohol. Our main objective was to go deeper into the role of the opioid system and the amniotic fluid, but also to start another line of research based on ethanol metabolism. All of this has been tested in neonatal and infant rats using different evaluation techniques and using ethanol but also non-alcoholic substances, such as vanilla flavor, and drugs, such as opioid receptor antagonists and an ethanol metabolism inhibitor.

The thesis starts with an Introduction where we review the basic topics needed to address this dissertation. First, we describe the prenatal development of various senses, paying particular attention to how and when the fetus starts to perceive chemosensory stimuli. This fact is relevant, given that the aim is to understand how the fetus learns about the stimuli present in their environment (amniotic fluid). Later, we review the available evidence relating to prenatal learning about flavors. Following this chapter about prenatal development, we present an introduction to ethanol, in which we explain

what alcohol is, how it is metabolized by our organism and the fetuses, and also the effects that it has on the reproductive system. Having explained prenatal development along with the general aspects of ethanol, we address the topic of the effects of prenatal exposure to alcohol. Within this third part of the introduction, we review the most recent publications showing statistical data about both the effects of exposure to ethanol on early development and the phenomenon of *Fetal Alcohol Syndrome*. Then, we explain the different behavioral effects that have been found following prenatal exposure to ethanol; most of them described in previous theses from this same laboratory (by Dr. Carlos Arias and Dr. Elena Díaz-Cenzano). To close the introduction, we describe two of the mechanisms that we believe play a very significant role in the prenatal ethanol exposure effect - the stimulation of the opioid system and the effects of acetaldehyde (first metabolite in ethanol oxidation). In both cases, we review the general aspects and their implications for behavior related to ethanol along with the relevant literature concerning fetus, neonate, and infant rats.

Following the introduction, we include another chapter about the importance of this study, in which we describe the objectives, the hypotheses, and also the expected results. Following this, we turn to an explanation of the General Methodology, describing all the evaluation procedures used in the different experiments along with the techniques used for the data analyses. In total, we conducted 7 experiments, each

of which are explained by specifying their objectives, hypotheses, particular procedures and results.

To finish the thesis, there is a conclusion chapter in which we will highlight the important results obtained in the different experiments along with a discussion that relates them to the current literature on the topic. In addition, we will review the limitations of the project and highlight any remaining or new questions that have emerged as a consequence of this work.

PRENATAL DEVELOPMENT

In mammals, prenatal development is divided into two main phases: the embryonic phase and the fetal phase. The embryonic phase starts after the fertilization of the ovum by the sperm cell and with the division of the zygote into smaller cells. The embryo is then implanted in the uterus and the growth starts slowly with the creation of the placenta and the umbilical cord. It is in this phase when the embryo acquires the characteristics of the specie, with the development of the bone marrow, bones, fingers, legs, and also the differentiation of the internal and external genitals. Upon becoming a fetus, the embryo already has all the internal organs formed, including the brain, and they grow faster during this second and last phase (Smoak, Roa and Rojas, 2014).

In humans, prenatal development takes approximately 38 weeks from the point at which the egg cell is fertilized by the spermatozoa. After fertilization, the cells start dividing until day 7, when the implantation process begins. This embryonic phase lasts until week 8, when the embryo is officially considered a fetus until the baby is born. In our experimental animal model - the rat - the process takes only 22 days: the pre-implantation period takes about 2 or 3 days, while the embryonic phase lasts until gestational day (GD) 15, when it is considered a fetus until the onset of birth on GD 22 (Smotherman & Robinson, 1988).

To understand how the fetus interacts with the environment and how the environment affects the fetus, it is important to analyze the context that surrounds the fetus during prenatal development. During this process the mammal embryos and fetuses develop in an aqueous environment, surrounded by the amniotic fluid and protected by two concentric membranes: the chorion and the amnion, inside the mother's uterus. The placenta is the organ that connects the developing fetus to the uterine wall to allow nutrient uptake, waste elimination, gas exchange via mother's blood supply, fight against internal infection and the production of hormones to support pregnancy. The fetus is connected to the placenta by the umbilical cord, whilst the latter connects with the mother through a vein and two arteries that are responsible of transporting all the nutrients to the fetus and all the wastes of the fetus back to the mother. The fetus lives inside the amniotic sac, surrounded by the amniotic fluid. This fluid represents one of the most complex, dynamic, and variable contexts of the prenatal environment. It is produced by the maternal plasma and diffused by the placenta. It contains all the necessary nutrients such as oxygen, glucose, lactic acid, fatty acids and amino acids (Liley, 1972). Moreover, in addition to these basic nutrients, the amniotic fluid receives different substances from the mother's diet, such as safe substances with chemosensory properties, but also potentially toxic substances such as analgesics, ethanol, and other drugs with pharmacological effects

(Robinson & Mendez-Gallardo, 2010). Even though the placenta is considered as a protection to the fetus, it is not a completely impermeable barrier. Substances from the maternal diet may pass through the placenta to the fetus depending on a range of factors, including the chemical properties of the substance, the concentration of the substance, and also the structural characteristics of the placenta (Robinson & Méndez-Gallardo, 2010). It is accepted that molecules of less than 600 Daltons can cross the placenta by simple diffusion, with no need for active transporters. In addition, the placenta suffers some structural changes in the middle of the gestational period. In particular, the membranes become thinner, making the placenta more permeable to some drugs (Unadkat, Dahling, and Vijay, 2004). In summary, it is not only the nutrients and flavored substances consumed by the mother that reach the amniotic fluid, but also drugs, viruses, and antibodies present in the mother's system (Robinson & Méndez-Gallardo, 2010).

Sensory development

Mammals can be classified as altricial or precocial animals, depending on their motor and sensorial maturation and the necessity of the mother to survive as neonates. Humans, primates and the majority of rodents are considered altricial due to their sensory immaturity and their dependence on the mother to receive food and care (Hall & Oppenheim,

1987). Despite the differences between species, the senses develop in the same order in all mammals - touch, proprioception, vestibular function, smell, taste, audition and vision. In the specific case of the rat development it follows the same order, but the last two senses are not completely functional until two weeks after birth. Thus, the rat neonate is blind and deaf until postnatal day (PD) 14 (Pedersen & Blass, 1982).

In reference to the somatosensory system (touch and proprioception), there is evidence showing its functionality before birth (Narayanan, Fox, and Hamburger, 1971). In this study, the authors observed that the fetuses were able to respond to the stimulation of some sensitive areas with localized reflexes or with a generalized motor activity. They also showed that the perioral region is the first area to acquire tactile sensitivity, and is a highly sensitive region during the remainder of the gestation period. There are several evidences, both in humans and rats, showing that there is a stable quantity of cyclic spontaneous motor activity in the fetus (Robertson, Smotherman, and Robinson 1990; Smotherman, Robinson, and Robertson, 1988) and that this early movement may be the first motor activation regulating the future perceptual exploration as neonates (Robertson & Bacher, 1992).

The perception of chemosensory stimuli by the fetuses has been regularly analyzed and finally confirmed to be

functional before birth. More specifically, it is known that the rat is able to perceive and also to respond to odors even before birth, since the olfactory system is relatively mature at that point (Pedersen, Shepherd, and Greer, 1987). This system is composed of three sub-systems: the olfactory bulb and glomerular complex which are completely mature and functional before birth, and the main principal olfactory bulb, which ends its maturation by the first days of postnatal life. The first sub-system allows the fetus to detect different odors dissolved in the amniotic fluid while the second permits the detection and recognition of the odors present in the neonatal/maternal environment right after parturition and during the lactation phase (Mistretta & Bradley, 1986). The earliest evidence of the functionality of the olfactory system demonstrated that the fetus recognizes the odors from its prenatal environment presented later after birth. For example Teicher and Blass (1977) described how newborn rats were attracted by the amniotic fluid odor and how this odor helped them to initiate their first suckling experience. Moreover, these same authors discovered that when the nipples of the mother were cleaned right after parturition, the newborns had problems in finding the nipple and initiating suckling (Teicher & Blass, 1976). Therefore, all these studies highlight the importance of olfaction in mammals as a critical sense to guide the newborn to the mother in their first hours as neonates (Hudson & Distel, 1983; Vince & Ward, 1984; Pedersen, Greer, and Shepherd, 1986; Schaal & Orgeur, 1992;

Robinson & Smotherman, 1994; Robinson & Mendez-Gallardo, 2010).

In the case of taste it has been observed that infant rats on PD 6 have a low percentage of taste buds that are functional and most of them mature after birth, and becoming completely mature on the 4th postnatal week just before adolescence (Coopersmith, Lee, and Leon, 1986; Mistretta & Bradley, 1986). However, evidence from more recent studies have demonstrated that the taste system starts to function before birth even though it is not completely mature. For example, there is ample data to support the notion that near-term fetuses are able to perceive and respond to sweet tastes (Arnold, Robinson, Spear, and Smotherman, 1993; Mickley, Remmers-Roebes, Crouse, Walker, and Dengler, 2000). Moreover, salt deprivation during development reduces the postnatal physiological responses to the taste (Przekop, Mook, and Hill, 1990). It has been hypothesized that even though the buds are not functional in the fetus, the chemosensory stimuli present in the amniotic fluid may directly stimulate the gustative nerve, thereby enabling the detection of tastes within the uterus (Mbiene & Farbman, 1993).

In reference to the auditory system, there are studies that have clearly established that in the final gestational trimester, the human fetus has an auditory system sufficiently developed to perceive sounds applied in the mother's abdomen (Moon & Fifer, 2000). Moreover, human neonates

are able to discriminate the sounds perceived during gestation and recognize the mother's voice (Hepper, Scott, and Shahidullah, 1993; Graven & Browne, 2008). Finally, the development of vision starts in humans in gestational week 26 and ends approximately 6 months after onset (Moon & Fifer, 2000). However, as mentioned before, these last two senses do not develop in the rat until PD 12 (auditory canal opening) and PD 13 (palpebral fissure opening) (Cornwell-Jones & Sobrian, 1977). For this reason, the near term rat fetuses and neonates depend on the tactile, odor, and taste senses to both learn about and interact with the environment.

Prenatal chemosensory learning

Since the topic of this thesis is concerned with prenatal experience with ethanol - a substance with chemosensory properties - it is important to review the available evidence related to flavor learning in prenatal animals. As mentioned before, it is known that fetuses perceive odors and tastes in utero, and also that this first experience with odors plays a significant role in the first postnatal days in helping the pup to recognize the mother's odor and also to find the nipple (Robinson & Mendez-Gallardo, 2010). Further, the near-term fetus displays ingestive behaviors such as mouth movements and swallowing, and therefore amniotic fluid gets into the mouth, providing the opportunity to learn about the stimuli present in the amniotic fluid (Chotro & Spear, 1997; Robinson

& Mendez-Gallardo, 2010). There are several studies showing that the fetus perceives not only the odors, but also learns about them, and that this learning modulates the response to the odor later in postnatal life (Abate, Pepino, Dominguez, Spear, and Molina, 2000; Diaz-Cenzano, Gaztañaga, and Chotro, 2014). For example, it is known that exposure to an odor on the last days of gestation induces an increased consumption of the substance later in adolescence (Abate, Spear, and Molina, 2001a) and in adulthood (Smotherman, 1982). This effect has been found in different mammal species (see table 1): In rabbits with prenatal exposure to juniper-berries (Bilko, Altbacker, and Hudson, 1994); in sheep using oregano (Simitzis, Deligeorgis, Bizelis, and Fegeros, 2008) and citrus (Schaal, Orgeur, and Arnould, 1995); in kittens with vanilla odor (Hepper, Wells, Millsopp, Kraehenbuehl, Lyn, and Mauroux, 2012); dogs exposed to anise (Hepper & Wells, 2006); in piglets with anise or garlic odors (Langendijk, Bolhuis, and Laurensen, 2007); in rats with apple juice (Smotherman, 1982a), citral (Pedersen & Blass, 1982a), garlic (Hepper, 1988) and cineol (Abate et al., 2000); as well as in humans with prenatal exposure to carrot juice (Mennella, Jagnow, and Beauchamp, 2001), garlic (Hepper, 1995) and anise (Schaal, 2000). All these studies show how prenatal exposure to different flavors via the maternal diet can modify the response

Specie	Exposed taste/odor	Prenatal exposure	Evaluation day	Results	Authors/Year
Rabbit	Juniper-Berries	GD 15 – PD 27(lactation) Maternal diet	PD 28	Preference for juniper-berries	Bilkó et al., 1994
Sheep	Oregano	GD 50 – GD 130 Maternal diet	PD 45	Preference for food flavored with oregano	Simitzi et al., 2008
Sheep	Citrus	Last 2 weeks Maternal diet	PD 1	Preference for amniotic fluid + citrus than to amniotic fluid alone	Schaal et al., 1995
Piglets	Anise and Garlic	Last month + lactation Maternal diet	3 rd and 7 th days after weaning	More acceptance of food in an anise odor environment during weaning	Langendijk, et al., 2007
Kittens	Vanilla	All pregnancy Maternal diet	48 h of birth 9-10 week 6 months	Preference to vanilla flavored food in the three tests	Hepper et al., 2012
Dogs	Anise	All pregnancy + lactation Maternal diet	10 weeks	Preference for aniseed food in a 2 choice test	Hepper & Wells, 2006
Rat	Apple Juice	GD 20 Injection to the	PD 60	Increased intake of apple juice in a 2 choice	Smotherman, 1982a

amniotic fluid			test		
Rat	Citral	GD 20 Injection to the amniotic fluid	PD 1	More approximation to the nipple with citral	Pedersen & Blass, 1982a
Rat	Garlic	GD 15 – GD 21 Maternal diet	PD 12	Preference for garlic comparing to onion	Hepper, 1988
Human	Garlic	Last month Maternal diet	15 – 28 hours after birth	Preference to garlic odor in a 2 odor choice	Hepper, 1995
Human	Carrot Juice	Last trimester + 2 months of lactation Maternal diet	5 th postnatal month	Less negative facial reactions to cereals with carrot than to cereals with water	Mennella, et al., 2001
Human	Anise	Maternal diet	PD 1 and 4	Less negative facial expressions and more movements with the head towards anise oil than to oil only	Schaal et al., 2000

Table 1: Studies conducted in different animal models and humans in which fetuses were exposed to a non-ethanol flavor without an explicit reinforcer and the postnatal response to those substances was evaluated.

to those flavors at different moments of postnatal life, increasing its consumption or acceptance compared with non-exposed flavors. This could be due to the effects of mere prenatal exposure or habituation to neophobia, but in any case the result is not only an increased acceptance but also a solid memory that can be retained throughout time, modulating the experience that these subjects will have later in life with those same stimuli.

In addition to the mechanisms of familiarization by mere stimulus exposure or habituation to neophobia, studies in recent decades have demonstrated how the near-term fetuses are able to learn by association between a conditioned stimulus (CS) and an unconditioned stimulus (US); i.e. classical conditioning. For instance, Spelt(1948) demonstrated conditioning to vibrotactile stimuli in the human fetus, whilst some years later other authors also found olfactory and gustatory conditioning (DeCasper & Fifer, 1980; Fifer & Moon, 1994). However, most of the Pavlovian conditioning works in fetuses were conducted in rats. Using apple juice as the CS, Stickrod, Kimble, and Smotherman (1982) provided the first evidence of a taste/odor conditioned aversion in rats. The flavor was injected on GD 20 directly into the amniotic sac of each pup, and 5 minutes later lithium chloride (LiCl) was also injected intraperitoneally (IP) to each experimental pup. Then, subjects were evaluated on PD 16 and the results showed a significant decrease in apple juice intake compared to the non-conditioned subjects. Smotherman and Robinson (1982b)

then reported further examples of taste/odor aversions in which they demonstrated that associative learning in uterus was possible starting on GD 17. They aversively conditioned menthol on GD 17 and two days later (GD 19) subjects demonstrated a conditioned suppression of the general activity in the presence of the conditioned stimulus. This response, however, was not found in fetuses not exposed to the flavor. Other results from the same investigation indicated that from GD 19 the rat fetus has the capacity to discriminate between two chemosensory stimuli (milk and lemon) exposed prenatally, modifying their facial responses towards those stimuli (1987). Another study has shown that the administration on GD 18 and GD 19 of garlic together with LiCl induces a conditioned aversion to that flavor evaluated 6 weeks after birth, during adolescence. However, this effect was not found when the prenatal administration was conducted earlier on GD 15 and 16 (Gruest, Richer, and Hars, 2004a). In addition to these studies, there is also other evidence of prenatal conditioning with alcohol exposure on late gestation (Arias & Chotro, 2003; 2005) but these results will be addressed in another chapter of this introduction.

Finally, it has been demonstrated that fetuses are able not only to acquire a conditioned aversion but also to show latent inhibition. This effect consists of the retardation of conditioning due to the non-reinforced preexposure of the stimulus to be conditioned (Lubow & Moore, 1959), and it is not easy to find in preweaning rats due to their immaturity

(Gaztañaga, Aranda, Díaz-Cenzano, and Chotro, 2014; Revillo, Gaztañaga, Aranda, Paglini, Chotro, and Arias, 2014). However, there is one report of this effect in the rat fetus (Mickley et al., 2013). In this study, the authors administered allicin directly into the amniotic sac of each pup on GD 18 and a day later, on GD19, they gave another exposure to allicin but this time paired with a LiCl injection. This was followed by a test on GD 21 in which they evaluated the hedonic responses of the fetuses. Subjects in the preexposed group did not show an aversion to allicin, while subjects not preexposed on GD 18 showed a reduction in the hedonic responses to the allicin, showing that they were effectively conditioned to the flavor.

To sum up, it is clear that the near-term fetus is able to perceive and learn about flavors present in the amniotic environment, most of which are derived from the maternal diet. This exposure to flavors has been found to modify the response of the neonates and infants to the same flavor, in some cases increasing its consumption or showing a preference compared to other flavors. Moreover, there is evidence to show how fetuses are capable of acquiring and expressing a conditioned response by the association of a flavor (CS) with a US.

ETHANOL

Ethanol, ethylic alcohol, or simply alcohol is an organic chemical compound with a relatively simple molecule with two carbon atoms, one oxygen atom and six hydrogen atoms ($\text{CH}_3\text{-CH}_2\text{-OH}$). It is derived by decomposition of vegetal carbohydrates, a process that can be spontaneous but that is accelerated by the catalytic action of yeasts (*Saccharoyces cerevisiae*). It does not contain any nutritive value for the organism, providing only 7.2 kcal per gram, and is regarded as empty calories (Lorenzo, Ladero, Leza, and Lizasoain, 1999).

Ethanol is not a normal product of human metabolism, except for the small amount of alcohol produced by the fermentation of the carbohydrates by the bacterial flora in the intestine, and it normally reaches our tissues by the ingestion of alcoholic beverages such as beer, wine, vodka, whiskey, etc. The ingestion of elevated amounts of ethanol interrupts the body's balance, since the organism needs to activate resources to processes it and eliminate it (Lorenzo et al., 1999). Its physical qualities make ethanol a very harmful substance for the organism: it is very soluble in water, which allows it to reach every single cell of the organism, and it is also soluble in fat (even though ten times less than in water), a reason why it crosses all the lipid barriers of our organism. Starting at concentrations considered to be normal for ingestion, it is a very toxic drug. When ingested, ethanol is rapidly absorbed by

the stomach and intestine for distribution throughout the body, although a small loss occurs by perspiration or urination. However, the absorption rate depends on different variables such as whether food has been ingested recently, age, sex, weight and metabolism of the person. For example, because of the difference in water proportion with respect to body weight, which is lower in women (44-55%) than in men (55-65%), the volume of distribution of ethanol is lower in women than in men, i.e. the concentration of ethanol in blood in women is higher than in men, with equivalent weight and ethanol consumption. Depending on all of these factors, ethanol will take from a few minutes to approximately an hour to reach the brain and produce the typical effects expected by the users. When the amount of ethanol exceeds the capacity of the organism to metabolize it, an "intoxicating effect" occurs, producing a variety of symptoms in the user, and also producing "hangovers", which arise from the difficulties for the organism to detoxify the system (Jung, 2001).

Absorption, distribution and metabolism

As mentioned above, ethanol is absorbed in the digestive system, passing through the stomach to the intestine or from the colon to the blood. The absorption of the drug depends mainly on the dose consumed and its concentration. For example, the main absorption occurs when the drug is in concentrations between 15 – 30 %. Taking into account the

individual differences and factors mentioned above, the peak levels of ethanol in the blood are usually reached within an hour after drug consumption. Following absorption, the drug is rapidly distributed to all the tissues of the organism, crossing the brain-blood barrier or the placenta with relative ease.

Even though a small part of ethanol is neutralized in the stomach before reaching the systemic circulation, at least 90 % of the ethanol consumed is metabolized by a process that takes place in the liver. This process consists of two enzymatic steps: the first of which is to convert ethanol into acetaldehyde and the second of which converts acetaldehyde into acetate.

From ethanol to acetaldehyde

To complete the first step there are 3 different enzymatic systems: alcohol dehydrogenase (ADH), the oxidative microsomal system (MEOS) and the catalase system (Escarabajal, 2002; Crabb & Liangpunsakul, 2007). However, not all of these systems play the same role in metabolization, the first of these being ADH, which converts 90% of ethanol into acetaldehyde. This enzyme is present in the liver in 17 different forms or isoenzymes, and their activation depends on the amount and concentration of ethanol. The rate of metabolization depends on the quantity of enzymes available and is independent of ethanol concentration, the ethanol oxidation and acetaldehyde levels being constant in the blood. As an example, it is believed that in a healthy sporadic drinker

the metabolization rate of ethanol ranges from between 60 and 150 mg/kg/hour (Escarabajal, 2002; Zakhari, 2006).

In the case of the MEOS, it is responsible only for 5 or 10 % of the total metabolic process, and its activity is enhanced when the concentration of ethanol in blood is very high. Moreover, this system also participates in the metabolism of other drugs, such as barbiturics, this being the reason why some alcoholics show resistance to the effects of this and other drugs (Escarabajal, 2002; Zakhari, 2006). In reference to the catalase system, there are abundant catalases in the liver that protect it from hydrogen peroxide. However, the quantity metabolized by this system is irrelevant, and it has been demonstrated that its inhibition does not affect the rate of peripheral ethanol oxidation.

In addition to the metabolization process that takes place in the peripheral system, it has been shown that the central nervous system also has all the necessary enzymes to metabolize ethanol. This fact was found when the researchers realized that acetaldehyde could be found in the brain after ethanol intake. Given that acetaldehyde does not cross the blood-brain barrier, not at least until it reaches very high concentrations in the periphery, the researchers in recent decades have been searching for the alternative pathways that the central nervous system uses to metabolize ethanol. Even though the enzymes ADH and the MEOS system have been detected in the brain, more recent data show that the

catalases are primarily responsible for the conversion of ethanol into acetaldehyde.

From acetaldehyde to Acetate

In the second step of ethanol's metabolic process, acetaldehyde from the peripheral but also from the central system is converted into acetate by the action of the enzymes aldehyde dehydrogenase (ALDH). Following this step, acetate is converted into water and carbon dioxide and eliminated from the organism.

Ethanol and the reproductive system

Among males, consumption of ethanol lowers only temporarily levels of the hormone testosterone. However, in frequent alcohol drinkers sexual impotence and impaired performance have been found, and even though they are able to participate in sexual activity, the addiction leads to a reduction in seminal fluids that could reduce fertility (Cicero, 1982; Van Thiel, 1983).

In females there are studies showing how ethanol blood levels vary considerably during the menstrual cycle in humans, being higher during ovulation and immediately before menstruation (B.M. Jones & Jones, 1976). However, there are other important variables that also affect the levels of ethanol in blood during the reproductive period, such as the

age and the nutritional state of the subject. It is known that the ethanol level in blood augments with aging due to the gradual decline in the proportion of water in the body (Abel, 1979). Further, a deteriorated nutritional state is easy to find among people suffering from alcohol addiction, which aggravates the toxic effects of the ethanol whilst having critical consequences for the reproductive system and in the development of the fetus (Abel & Dintcheff, 1984). More specifically, in alcoholic women ethanol disrupts the normal menstrual cycle producing irregular menstruations or even a complete cessation, absence of ovulation, and infertility (Mello, Mendelson, and Teoh, 1993; Emanuele, Wezeman and Emanuele, 2002).

During pregnancy, studies with rats have shown that after ethanol administration the levels of ethanol in blood are higher in pregnant rats than in non-pregnant rats (Abel, 1984; Aber & York, 1979; Sanchis & Guerri, 1986). However, it has been found that during pregnancy the rate of ethanol elimination is incremented and as a consequence, the ethanol concentrations in different tissues are lower, which in some way attenuates the teratological effects of ethanol (Bedger, Hidestrand, Shankar, McGuinn, and Ronis, 2005). This can be explained by the discovery of higher levels of gastric ADH and ALDH enzymatic activity in pregnant rats (Da-Silva, McLean, and Beales, 1996). In particular, it has been shown that during gestation ALDH activity is higher together with the accumulation of acetaldehyde (Petersen, Panter, and Callins,

1977). Moreover, other studies have shown that with the same ethanol dose the acetaldehyde level in pregnant rats is superior compared with non-pregnant rats. For example, with an administration of 3 g/kg of ethanol on GD 20 the concentration of acetaldehyde in blood was four times higher than in a non-pregnant rat (Zorzano & Herrera, 1989a). Further, in a later study the same authors showed that administration of 10 mg/kg of acetaldehyde to pregnant rats produced a higher concentration of this metabolite compared with virgin rats (Zorzano & Herrera, 1989b). It is not clear, therefore, whether the metabolic changes associated with gestation attenuate the teratogenic effects of ethanol and acetaldehyde.

During lactation, ethanol elimination rate is also augmented but in this case the augmentation is not related to a higher activity of ADH (Da-Silva et al., 1996), since several studies have shown that the capacity to metabolize acetaldehyde is diminished in lactating rats compared with non-lactating female subjects (Gordon, Baraona, and Lieber, 1985; Gordon, Baraona, Miyakawa, Finkelman, and Lieber, 1985).

Metabolism of ethanol in the fetus

Ethanol ingested by the mother during pregnancy crosses the placenta with ease (Guerra & Sanchis, 1985) and is

rapidly distributed to the fetal tissues to later accumulate in the amniotic fluid (Hayashi, Shimazaki, Kamata, Kakiichi, and Ikeda, 1991; Nava-Ocampo, Velazquez-Armenta, Brien, and Koren, 2004). Data suggest that the fetus eliminates ethanol via maternal hepatic metabolism (Szeto, 1989) due to the immature state of ADH enzymes before birth (Sanchis & Guerri, 1986). Because of this, and even though blood concentrations are similar in the fetus and the mother, ethanol takes longer to be eliminated from the amniotic fluid than maternal blood. Therefore, several studies have found ethanol in the fetus at a time after the maternal blood was free of ethanol (Hayashi, 1991; Nava-Ocampo, Velazquez-Armenta, Brien, and Koren, 2004). Acetaldehyde levels in fetal blood do not reach significant levels due to the protection mechanism of the placenta (it contains ALDH enzymes to convert acetaldehyde in acetate). However, when the concentration of the maternal acetaldehyde is very high and the placental capacities are overcome, some studies have detected this metabolite in fetal blood (Espinete & Argiles, 1984; Zorzano & Herrera, 1989a). For example, in one study acetaldehyde was not detected in either fetal blood or amniotic fluid after an administration of 4 g/kg of ethanol to the mother (Zorzano & Herrera, 1989a). On the contrary, other authors found in two different studies small levels of acetaldehyde in fetal blood after 3 g/kg of ethanol administration to the mother (Gordon et al., 1985). Similarly, in a third study acetaldehyde was detected in fetal blood even though the level was ten times

lower than in maternal blood (Hayashi, 1991). Acetaldehyde was also found in fetal blood after maternal administration but at 40-70 % of maternal acetaldehyde levels (Guerra & Sanchis, 1985). Other authors have hypothesized that acetaldehyde can be detected in fetal blood when the concentration in maternal blood is higher than 80 μM (Zorzano & Herrera, 1989b). Moreover, they confirmed that even though the activity of the enzyme ALDH (which metabolizes acetaldehyde) is much lower in the fetus than in the mother, it is present in the placenta, in the fetal liver, and the fetal brain. Finally, other studies have shown that ALDH can be detected from GD 15, but is not mature until PD 20 (Sjoblom, Pilstrom, and Morland, 1977; Hollstedt & Rydberg, 1985).

PRENATAL EFFECTS OF ETHANOL EXPOSURE

Ethanol during early development

Alcohol use during pregnancy is known to cause severe damage to the embryo and the fetus. As the World Health Organizations stated on the *Guidelines for identification and management of substance use and substance use disorders in pregnancy* (2014) *"the use of alcohol, illicit drugs and other psychoactive substances during pregnancy can lead to multiple health and social problems for both the mother and child. Use of alcohol during pregnancy can lead to fetal alcohol syndrome and other harm such as spontaneous abortion, stillbirth, low birthweight, prematurity and birth defects"*.

Even though worldwide organizations claim the potential negative effects that alcohol exerts, data suggest that women still consume alcohol during pregnancy (Rausgaard, Ibsen, Jorensen, Lamont, & Ravn, 2014; O'Keeffe et al., 2015; Cannon, et al., 2014; Polanska, Jurewicz, & Hanke, 2015; Blasco-Alonso, et al., 2015). It is important to note, however, that most women discover their pregnancies weeks after the onset of gestation, and therefore unknowingly maintain their regular alcohol consumption until being aware of their condition. For this reason, it is common to find more fetal alcohol exposure during the first weeks of gestation with the trend decreasing significantly thereafter (Blasco-Alonso, et al., 2015; Polanska et al., 2015). However, some women still

consume alcohol - at least in very low doses. For example, a recent study carried out in Denmark showed that 3.6% of women had a positive urine sample for ethanol at 12 weeks of gestation (Rausgaard, et al., 2014). In Spain a recent study has found that almost 40.7% of the women had consumed alcohol in the first trimester, 23.1 % in the second trimester, and 17.1% in the third trimester (Blasco-Alonso, et al., 2015).

Prenatal teratological effects

Alcohol or ethanol consumption during gestation can produce severe damage to the fetus (E.L. Abel, 1999a; 1999b). Curiously, this fact has been described in various ancient texts such as in the Bible and in some of the papers written by Aristotle. However, it was not scientifically described until 1968, when a group of investigators systematically reported the damage provoked by prenatal exposure to ethanol (Lemoine, Harousse, Borteyru, & Menuet, 1968). This initial work on the topic was conducted with 127 children of alcoholic mothers, and all of them showed common characteristics such as developmental retardation, cranio-facial alterations, hyperactivity, mental retardation, and psychomotor alterations. Moreover, this was the first study suggesting a correlation between the different damage levels and maternal alcoholism level. A few years later, Jones and Smith (1973) described a common path of malformations in 8 children born from alcoholic mothers, and they labeled it as Fetal Alcohol

Syndrome (FAS). They divided the characteristics of the FAS into three main groups: 1) Deficiency in prenatal and postnatal development, 2) cranio-facial alterations, and 3) dysfunctions of the central nervous system. Since the inception of FAS and the identification of three characteristic categories, the scientific community began to reveal new cases whilst also searching for answers using animal models, which demonstrated the potential teratogenic effects of ethanol.

The criteria needed to diagnose complete FAS are:

- Deficit in both prenatal and postnatal development. The prenatal deficit is related to the weight or/and height of the fetus and the baby, and it has to be below the 10th percentile that corresponds to the age.
- Evidence of dysfunctions in the central nervous system, such as neurobiological and cerebral abnormalities, and a delay in psychomotor development and intellectual impairments.
- Specific cranio-facial abnormalities: microcephalia, short palpebral fissures and in the premaxillary zone (e.g., flat upper lip, flattened philtrum and flat midface).

Depending on factors such as the mode of consumption and the dose, exposure to this drug can produce different levels of malformations in the fetus, which can range from very mild effects after sporadic ethanol intake to severe effects in

alcoholic mothers, which will probably fit with FAS diagnostic criteria. In fact, children with a complete diagnosis of FAS are born from chronic alcoholic mothers (Streissguth, Barr, & Martin, 1983). Nevertheless, only 30-45% of the children of these mothers showed the whole diagnostic criteria and the remainder of the children were not diagnosed because they only presented partial symptoms. To counter this problem, in 1997 the National Institute of Medicine of United States (NIM) defined five diagnostic characteristics of FAS (Cancino & Zegarra, 2003):

- 1- FAS with confirmation of maternal ethanol consumption.
- 2- FAS without confirmation of maternal ethanol consumption.
- 3- Partial FAS without confirmation of maternal ethanol consumption.
- 4- Neonatal disorders caused by ethanol.
- 5- Neurodevelopmental disorders related to ethanol.

This new description, however, was not sufficient to explain the variability of effects and symptoms resulting from fetal alcohol exposure. Thus, children with only partial characteristics of FAS were being inadequately diagnosed and were not receiving the appropriate treatment from mental health professionals (Kable et al., 2015). To give a definite answer to the problem, almost a decade later, in 2004, governmental, research and advocacy organizations

sanctioned discussions concerning Fetal Alcohol Spectrum Disorders (FASD; Warren, & Murray, 2013). This concept, FASD, is *“an umbrella term describing the range of effects that can occur in an individual whose mother drank alcohol during pregnancy. These effects may include physical, mental, behavioral and learning disabilities with lifelong implications”* (Chudley, Conry, Cook, Loock, Rosales, & LeBlanc, 2005). The concept of spectrum was introduced to include all the different variations that appear as a result of fetal alcohol exposure and that do not necessarily fit well with the FAS diagnosis. This allows professionals to diagnose neurodevelopmental disabilities associated with prenatal alcohol exposure (ND-PAE), even though there is no physical evidence (brain damage or facial abnormalities, for example) of FAS (Kable et al., 2015). The symptoms of ND-PAE have been clustered into three large domains: neurocognitive functioning, self-regulation, and adaptive functioning. The first domain refers to intellectual deficit, executive functioning, learning and memory problems, and visuospatial processing. The second domain refers to mood or arousal regulation and attentional and impulse control problems. Finally, the third domain focuses on deficits in communication and social skills in daily living activities and in motor functioning (Kable et al., 2015).

Prenatal behavioral effects of ethanol

As we have seen before, ethanol has very toxic effects on the fetus and can exert long-lasting symptoms in children. However, these mild effects usually appear after regular and high dose ethanol consumption by the mother, but this by no means discards the possibility that moderate to low doses or casual consumption do not have any behavioral effects. These behavioral effects are not related to brain damage or dysfunction, as occurs in FAS, but to learning about the substance, and specifically, modifying the response to it following prenatal exposure.

In humans, there are studies with 14-21 year olds that show a clear correlation between prenatal ethanol exposure to moderate doses of ethanol and later alcohol abuse related problems (Baer, Barr, Bookstein, Sampson, & Streissguth, 1998; Baer, Sampson, Barr, Connor, & Streissguth, 2003). These studies were based on interviews conducted with the pregnant mother in the middle of the gestation period, controlling alcohol and nicotine consumption. These families were followed up every 7 years from newborns and finishing at the age of 21, with the objective of evaluating consumption and alcohol related problems, registering the quantity and the frequency and the dependence level. The results showed a positive correlation between prenatal ethanol exposure and alcohol associated problems at the age of 21. The authors concluded that in their study the exposure to ethanol during

pregnancy was the best predictor of problems related to this drug - and better than other factors such as drug consumption or family history of alcoholism.

In addition to problems with alcohol abuse, it has been shown that prenatal exposure to this substance can modulate the response to ethanol throughout life (Mennella & Beauchamp, 1996). In one study, toys impregnated with ethanol were presented to the children and they found that children born from a mother that consumed this drug during pregnancy showed more interactive behaviors with those toys compared with toys impregnated with other odors (Mennella & Beauchamp, 1998). In another study it was found that newborns 1 or 2 days old, whose mothers were habitual consumers of moderate doses of alcohol during gestation, showed more reactivation and agitation to the ethanol odor, compared with newborns whose mothers were classified as non-drinkers (Faas, Sponton, Moya, & Molina, 2000). In the same study, the authors tested the response to a lemon odor but they did not find any differences between children with or without prenatal exposure to ethanol. This indicates that the neonates recognized the ethanol odor, which they had experienced in the amniotic fluid, and accordingly modified their response to this odor. Moreover, this responding was specific to the ethanol, since there was no generalization to other odors, as in the case of lemon (Faas, et al., 2000). A recent study has demonstrated that 1 or 2 day old neonates given prenatal experience with ethanol showed no

teratological effects but attributed more hedonic value to ethanol's chemosensory properties, showing, for example, more appetitive responses (Faas, March, Moya, & Molina, 2015).

The results of the experiments with animal models appear to follow the same path as that previously described for humans. The first relevant study with animals was published in 1976 with rats and they found that chronic exposure to ethanol throughout gestation induced hyperactivity along with a higher consumption of ethanol as an adult (Bond & Digiusto, 1976). These results, together with another study showing a correlation between maternal alcohol consumption, hyperactivity, and alcoholism in offspring (Cantwell, 1972), gave further support to the possibility that hyperactivity - and not prenatal exposure to the ethanol - was responsible for the predisposition to high alcohol consumption (Bond & Digiusto, 1976).

In subsequent decades, various studies were conducted with rats focused on these effects by administering ethanol to the mother throughout the whole of the gestation period. Various alcohol beverages were used for this purpose, such as beer, white wine, ethanol dilutions, etc. and the procedures also varied (ethanol as unique liquid in their diet, sugared alcoholic solutions...). Further, in some studies ethanol was administered in different gestational periods and different evaluation indexes were used. In spite of using these diverse experimental procedures, in all studies the common factor

was that experience with ethanol during pregnancy produced an augmentation in ethanol consumption or a heightened preference for ethanol (for revision see Chotro, Arias, & Laviola, 2007). And whilst different hypotheses have been proposed to account for these effects, it is generally accepted that the higher intake was related to the teratological damage produced in the neurotransmitter systems and the HPA axis. In particular, it has been pointed out that the alteration in the neurochemical system could be responsible not only for predisposition to ethanol abuse but also to other drugs.

Nevertheless, none of these studies considered the possibility of fetal learning as the mechanism underlying the increased preference or intake of ethanol. It should be noted that the increased ethanol intake after prenatal exposure is observed when the subjects are exposed to the substance during the whole gestation period (Youngentob, Molina, Spear, & Youngentob, 2007) and also when a moderate dose of the drug is administered a few days before birth (Molina, Chotro, & Dominguez, 1995). As explained in the chapter about sense development and prenatal learning, the fetus has the capacity to perceive and learn about chemosensory stimuli present in its environment. Thus, using this hypothesis as a starting point, a number of studies conducted in the last two decades have shed some light on this issue. Of central importance has been the need to analyze how experience in early ontogeny with the odor and taste of ethanol affects the postnatal response to this drug.

Chotro & Molina (1990) published a study showing that rat fetuses given brief experience with the odor/taste of ethanol developed a preference for its odor and also consumed more ethanol compared with control rats. The experimental procedure consisted of injecting ethanol directly into the amniotic fluid, approximately to the fetus facial area, 10 minutes before practicing a cesarean. The critical feature of this procedure is that it permitted the fetus to perceive the chemosensory properties of the drug whilst avoiding its pharmacological effects. The results demonstrate that the fetus was able to perceive ethanol and also to react differently to it after birth. Subsequent investigations confirmed these results and found that the same exposure to ethanol was sufficient to potentiate a conditioned preference when the ethanol odor was paired with an intraoral administration of sugar (Chotro, Cordoba, & Molina, 1991). The same study also presented data indicating that the conditioned aversion to ethanol was attenuated after prenatal experience with the drug. These results indicate that prenatal treatment with ethanol could potentiate the acquisition of a preference or the retardation of an aversion, concluding that the memories generated in uterus have an appetitive hedonic value.

Subsequent work demonstrated that the preference described previously was the result of an association between the chemosensory properties of ethanol and the stimulation of the fetus during the cesarean (Molina & Chotro, 1991). In this study, the fetus received ethanol 10 minutes prior to the

cesarean and exhibited a greater response to the ethanol odor than the fetus given equivalent exposure to the ethanol 40 minutes prior to the cesarean. The same outcome was observed with a lemon solution. Two other studies have confirmed these results and have proposed that stimulation during the cesarean could serve as appetitive unconditioned stimuli (US) associated with the odor of ethanol (Dominguez, Bocco, Chotro, Spear, & Molina, 1993; Molina & Chotro, 1991). During birth or cesarean there is an increase in the activity and it has been demonstrated that those stimuli that are able to activate newborns, such as morphine or amphetamine, anogenital stimulation, pressure in the tail, intraoral milk infusion, or exposure to warm temperatures, are effective appetitive USs (Coopersmith, Henderson, & Leon, 1986; Dominguez, Chotro, & Molina, 1993; Pedersen & Blass, 1982a). Therefore, the perception of ethanol inside the amniotic sac could be associated with the stimulation of the cesarean section, acting as a US. These latter studies confirmed that when ethanol is introduced into the amniotic fluid before birth, it is perceived by the fetus, which leads to subsequent changes in responding to the drug during infancy. The various authors suggested that the learning mechanism underlying this experience is the association of the chemosensory properties of ethanol with the effects of stimulation and activation of the fetus during birth or cesarean.

Following the discoveries previously reviewed, some researchers began to investigate the effects of prenatal

exposure to ethanol but only on the last days of gestation (days 17, 18, 19 and 20) through maternal intragastrical administration. In the majority of these studies the authors used a low or moderate dose of ethanol (1 or 2 g/kg), and a procedure referred to as "binge drinking" that produces a range of 40 to 100 mg% of ethanol in the blood and amniotic fluid. With this procedure, the fetus was exposed not only to the flavor of the drugs but also to its pharmacological effects. As explained in the previous chapter, ethanol crosses the placenta with ease, and is distributed to all tissues, reaching the amniotic fluid (Guerra & Sanchis, 1985; Szeto, 1989). In the first study of these characteristics, the infant rats that had been exposed to ethanol showed increased ethanol consumption two weeks after birth (Molina et al., 1995). Generalization of this response was also found to a solution imitating the bittersweet chemosensory properties of ethanol (Di Lorenzo, Kiefer, Rice, & Garcia, 1986). A further study found that the prenatal treatment was also modifying the response of neonates (or even fetuses) to the ethanol odor and not to other sensorial stimuli (Chotro, Kraebel, McKinzie, Molina, & Spear, 1996; Chotro & Spear, 1997; Dominguez et al., 1996; Dominguez, Lopez, & Molina, 1998). It is important to note that this prenatal ethanol exposure did not induce any teratogenic effects since there was no detection of underweight fetuses or newborns, or changes in the brain hemispheres (Dominguez et al., 1998). In another study using the same procedure, prenatal ethanol exposure was found to modify the motor response

and heart rate when ethanol was presented to the infant rats two weeks after birth (Chotro et al., 1996). All of these differential responses to the odor and taste of ethanol were interpreted as evidence of the hedonic and positive connotation of the prenatal experience, since the result was always an increased appetite for the substance.

In contrast with the results described previously, other experiments cast some doubts on this hypothesis (Abate, Pepino, Dominguez, Spear, & Molina, 2000; Abate et al., 2001). In those experiments ethanol was administered on the last days of gestation (days 17, 18, 19, 20) together with an odor by means of intragastric administration. The notion was that ethanol would serve as a US, while the odor (cineole) would act as a CS. On the basis of the results and explanations described previously, the researchers expected to find an association between both substances and therefore, to observe more responses to cineole. The results, however, showed that the group that had experienced paired presentations of both substances responded with less oral movements to cineole than the group given unpaired presentations of the stimuli. In another study using similar treatments, the group that received the substances together showed less cineol intake compared with the group that received both substances unpaired (Abate et al., 2001). The results obtained in the group that received cineole and ethanol separately (increased oral reactions and intake) were explained by the non associative process of familiarization, and the authors explained that

presenting both substances simultaneously is likely to hinder this learning process. The authors, however, concluded that due to the ambiguous results, the affective value of the memory acquired after prenatal exposure to the drug still remain unclear (Abate, Varlinskaya, Cheslock, Spear, & Molina, 2002).

Possible mechanisms underlying the prenatal ethanol exposure effect

Several studies have since been conducted working on the hypothesis of a conditioned preference. In particular, the chemosensory properties of ethanol would act as a CS as in previous studies, but some authors began to consider the possibility that the US could be the pharmacological properties of ethanol (Chotro & Arias, 2003). These pharmacological properties, according to the authors, would be mediated by the opioid system.

In addition, several recent studies have highlighted the importance of ethanol metabolism in the reinforcing effects of ethanol. For example, the first metabolite in the oxidation chain of ethanol, acetaldehyde, appears to be responsible for at least some of the effects that ethanol exerts.

In this thesis we have focused on studying the possible role of both of these systems - the opioid system and acetaldehyde, as possible mechanisms underlying the

reinforcing effects of prenatal ethanol exposure. Therefore, the following chapters will describe the general features of these systems along with their relationship with prenatal exposure to ethanol.

THE OPIOID SYSTEM

General aspects

To understand the reinforcing effects of ethanol, it is important to study the biochemistry and physiological processes of this drug in the brain. As stated in the previous chapter, there are no specific ethanol receptors in the central nervous system so it is accepted that the effects that it exerts are produced by the alteration of the endogenous opioid neural system (Gianolakis & Waele, 2004). Thus, the opioid system has not only been investigated with regard to examining the issues related to alcohol, but also in other fields, such as prenatal/neonatal learning, due to its critical role in the acquisition and expression of some conditioned responses in the fetus (Smotherman, 2002a; 2002b).

In total, more than 50 different neuropeptides have been identified in the brain. These substances are synthesized in the vesicles located inside the neurons' soma and they are carried through axonal transportation. The endogenous peptides have been classified into three different families: enkephalins, dynorphins, and endorphins. The first endogenous opioid peptides - the enkephalins - were discovered in 1976 (Hughes, Smith, Kosterlitz, Fothergill, Morgan, and Morris, 1975) and some years later two other peptide families were described - the endorphins and dynorphins (Bradbury, Feldberg, Smyth, and Snell, 1976;

Goldstein, Tachibana, Lowney, Hunkapiller, and Hood, 1979; Khachaturian, Lewis, Schäfer, and Watson, 1985; Bordner & Deak, 2015). These opioid peptides and their receptors are located in various central structures such as the spinal cord, pituitary gland, adrenal medulla, and the autonomous nervous system.

The opioid peptides and opiate drugs can act on different type of receptors, which have been divided into the following five different families: mu, kappa, delta, sigma and epsilon. However, mu, kappa and delta receptor systems are the most studied families due to their known implication in many processes of the organism. Mu receptors for example, are divided into two subgroups depending on their affinity for the agonists (mu1 and mu2, high and low affinity respectively) and can be found in many areas of the brain, such as in the thalamus, dorsal and ventral striate nucleus or in the amygdala and nucleus accumbens. They mediate analgesia, reinforcement and sedation, and their activation produces changes in food intake, inhibits intestinal transit, produces respiratory and cardiovascular depression, and has neuroendocrine effects (Bodnar, 2009). On the other hand, delta receptors are located in the neocortex, striatum nucleus, olfactory areas, substantia nigra or the nucleus accumbens. Their activation produces analgesia, reinforcement, sedation, changes in food intake whilst also inhibiting intestinal transit (Honkanen, 1999; Meririnne, 2002). Finally, kappa receptors are found in the nucleus accumbens, the hypothalamus, caudate

nucleus, olfactory areas, interpeduncular, paraventricular and supraoptic nucleus, amygdala and hypophysis (Honkanen, 1999; Meririnne, 2002). The activation of these receptors produces dysphoria, sedation, changes in food intake and effects in the neuroendocrine system (Bordnar, 2009). In general terms this neurochemical system contributes to thermal regulation and has effects on the cardiac, respiratory, muscular and immune responses (Kehoe, 1988). It also mediates affective states and participates in the acquisition of appetitive responses and the reinforcing processes of positive and negative stimuli. Moreover, it plays a significant role in the perceptive and attentional mechanisms, and modulates sensorial and motivational aspects of aversive and positive stimuli (Kehoe, 1988). An important characteristic of the endogenous opioid receptors is that in normal physiological conditions almost all of them are silent and do not show any activity (Herz, 1997).

Development of the opioid system

The first evidence of opioid peptides in the central nervous system of the rat is on gestational day (GD) 11. Endorphins are the first to appear and it is believed that they play an important role in fetal and postnatal growth, at least in rats (Herlenius & Lagercrantz, 2001). At GD 15 approximately 10 % of the total endorphins found in weaned rats can be detected in the fetus – a similar distribution to that found in

adults. However, the enkephalin and dinorphin percentages are very low at this same stage, emerging later in intrauterine development (Kehoe, 1988).

Even though the number of opioid receptors increases progressively during gestation, the effects of opioids depend on the maturation of the organism. Mu and kappa receptors are expressed at high levels in the rodent brain during early ontogeny (Houser & Mangoura, 1998). At birth, mu receptors reach 40 % of the total amount of the adult rat (Herlenius & Lagercrantz, 2001; Kehoe, 1988). On postnatal day 2 a high density of mu receptors is found in the striatum nucleus, olfactory tubercle and nucleus accumbens, but this density decreases with growth until they reach a distribution equivalent to that of an adult rat. Kappa receptors are also detected at birth at 65 % of the total level found in adult rats (Kehoe, 1988). In addition, on postnatal day 2 these receptors are localized in the olfactory tubercle and in the globus pallidus, and their density also decreases with the development of the organism. In contrast, delta opioid receptors are not extensively expressed before birth and they appear on the second postnatal week of the rat (Houser & Mangoura, 1998; Herlenius & Lagercrantz, 2001, 2004; Kehoe, 1988; Attali, Saya, and Vogel, 1990). Data suggest that mu and kappa opioid receptor levels are stable until PD 10 when a rapid increase takes place for 5 days, until PD 15, and begins to decrease until adulthood (Kehoe, 1988; Attali, Saya, and Vogel, 1990).

Learning and the opioid system

As stated previously, in rats it is clear that kappa and mu opioid receptors are functional during the second half of the gestation period and that stimulation of these have an effect on fetal behavior (Smotherman 2002a, 2002b). Several studies with rat pups have demonstrated that maternal milk activates the opioid system in the fetus inducing conditioned responses mediated by this neurochemical system. It has been shown, for instance, that the administration of milk to the newborn rat delivered by cesarean has antinocioceptive effects similar to those exerted by morphine (Blass, Jackson, and Smotherman, 1991; Smotherman & Robinson, 1992b). Moreover, the administration of an opioid antagonist (naloxone or naltrexone) impairs the effects produced by milk, confirming the role of the opioid system in this response (Blass et al., 1991). Further, it has been found that when administering lemon intraorally into the fetus, it reacts by displaying a behavioral response known as facial wiping, which consists of rubbing the cheeks using its forepaws, a response that is considered to be aversive. By giving previous administration of milk or injecting morphine, this response to lemon is prevented, an effect blocked by kappa opioid antagonists (Smotherman & Robinson, 1992b; 1992c). This same antagonist reduces another typical behavior of the fetus - the stretching response. This behavior is a representation of the sucking response and it appears when the fetus receives milk

directly to the mouth and it continues after birth during lactation (Smotherman & Robinson, 1992b).

In other studies it has been shown that a specific ligand of the kappa opioid receptor (dinorphin A) or natural fluids such as maternal milk, can act as a US, which if paired with neutral stimuli converts the target into a CS (Smotherman, 2002c; Smotherman & Robinson, 1994; Varlinskaya, Petrov, and Smotherman, 1996; Robinson & Mendez-Gallardo, 2010). For example, mere exposure to an artificial nipple (CS) does not activate the opioid system (Smotherman, 2002; Smotherman, Simonik, Andersen, and Robinson, 1993) but when its exposure is associated with the effects of dinorphin A or an intraoral administration of milk (US), the same artificial nipple subsequently produces a conditioned activation of the opioid system (Smotherman, 2002c; Smotherman & Robinson, 1994; Varlinskaya et al., 1996). Moreover, it is not only maternal milk that has the capacity to activate the opioid system in the fetus. Amniotic fluid and saliva (with one of its components, dimethyl disulfure) have also been shown to activate the same system and to affect fetus behavior in the same way as milk.

Some other studies have shown that soon after birth the association between a flavor and LiCl will generate an aversion or a preference depending on the dose of LiCl. With high doses an aversion is observed (as in adults) while with low doses a conditioned preference has been reported in 5-day old

rats (Kehoe, 1988). The administration of an opioid antagonist prevents both conditioned preference and aversions - apparently impeding the learning process - which indicates that the opioid system is somehow playing a modulatory role in conditioning. In contrast, the administration of a low dose of an agonist, such as morphine, has been shown to produce conditioned preferences to odors or flavors during the initial days after birth (Kehoe & Blass, 1986). Conditioned preference to an odor has been found in the neonatal period after pairing the odor with a tactile or an electrical stimulus, and this preference was also blocked by the administration of an opioid antagonist, indicating that it was mediated by this system (Roth & Sullivan, 2001; 2003). These same authors have suggested that the opioid system plays a key role in neonatal learning during a sensitive period that extends until PD 9 in which the neonates are able to rapidly acquire odor preferences (Roth & Sullivan, 2001; 2003). Therefore, on PD 8 conditioned odor preferences can be found even when in association with stimuli that induce some pain to the neonate, such as a shock. This same procedure, however, produces an aversion to the odor when applied on PD 12. This sensitive period is not due to a deficit in pain perception, and has also been found in a range of animal species including dogs, chickens, primates, and humans. The sensitive period terminates when the pups show a greater capacity to move and travel, i.e. when pups are able to move away from the nest, the aversive stimuli lose the capacity to induce conditioned

preferences (Roth & Sullivan, 2003). The authors hypothesized that this learning system may have been developed by altricial animals to ensure neonates maintain in contact with the mother even though the maternal care is not of good quality.

All these data suggest that the opioid system of the neonate plays a facilitating role in the acquisition or consolidation of olfactory preference learning and that it participates in the acquisition of the conditioned responses. This explanation could also be extended to the prenatal period since some studies have demonstrated that it is possible to condition the activity of the opioid system in the fetus, associating a neutral stimulus with the activation of the opioid system (Arnold, Robinson, Spear, & Smotherman, 1993).

Ethanol and the opioid system

As mentioned previously, to understand the reinforcing effects of ethanol it is necessary to review the important role of the endogenous opioid system, which modifies ethanol consumption either alone or through interaction with other neural systems such as the dopaminergic systems (Gianoulakis, 2001; 2004). The reinforcing system consists of a number of pathways connecting different brain structures, including the ventral tegmental area, nucleus accumbens, septal nucleus, amygdala, hypothalamus, hippocampus, and prefrontal cortex. The reinforcing effects of addictive drugs

are mediated chiefly by the mesolimbic dopaminergic pathway, which originates from the ventral tegmental area. In the specific case of ethanol, activation of the mesencephalic dopaminergic pathways is mediated by the activity of the endogenous opioid system (Koob & Volkow, 2010).

The activation of the opioid system has important effects on motivation and mood. In humans, the consumption of substances that activate this neurochemical system produces euphoria together with the compulsive need to continue consuming the substance. The development of addiction towards these substances appears to be related to the activation of the reinforcing pathways, which are mediated by the opioid system (Herz, 1997b; 1998). In general, the activation of mu and delta receptors is related to the acquisition of appetitive reinforcement, while activation of kappa receptors induces aversive states in adults.

In studies with ethanol, there is abundant evidence to confirm that the reinforcing effects of this drug are mediated by the activation of the opioid system. The administration of ethanol affects the expression of mu and delta receptor, producing different effects in animals and within different brain regions (Gianoulakis, 2001; Herz, 1997b). Further, several studies in vitro, in vivo with rats, and with a clinical sample of a population at high risk of alcoholism, have confirmed that the acute or chronic administration of ethanol affects the release of B-endorphins (Gianoulakis, 2001; Herz, 1997; Honkanen,

1999). This effect appears to be dependent on the dose; a low ethanol dose increases the release of opioid peptide while a high dose produces an inhibitory effect (Herz, 1997).

Further evidence supporting the participation of the opioid system in the reinforcing effects of ethanol comes from studies with opioid antagonists. Clinical studies and laboratory experiments have shown that opioid antagonists such as Naloxone, Naltrexone or Nalmefene, have effects on the withdrawal syndrome, decreasing drug consumption days and increasing the abstinence period (Rohsenow et al, 2000; Rohsenow, Monti, Hutchison, Swift, Colby and Kaplan, 2000; Rohsenow, Monti, Martin, Michalec and Abrams, 2000; Yeomans & Gray, 2002). It has also been demonstrated that the administration of an opioid antagonist decreases ethanol self-administrations (Altshuler, Phillips, and Feinhandler, 1980). Therefore, based on these and other findings from animal research, naltrexone has been successfully tested as a treatment for alcoholism in two clinical studies (O'Malley, Jaffe, Chang, Schottenfeld, Meyer, and Rounsaville, 1992; Volpicelli, Alterman, Hayashida, and O'Brien, 1992). However, the action mechanism of opioid antagonists in ethanol consumption still remains unclear and it is an issue of current debate. For example, there are questions surrounding the specificity of the action of these antagonists, since some authors suggest that they act on the basic mechanisms of food and water intake behaviors (Yeomans & Gray, 2002). Studies with animals have found that opioid antagonists decrease ethanol consumption

whilst in some cases they reduce the consumption of sugared substances (Cichelli & Lewis, 2002; Yeomans & Gray, 2002). In any case, it is also true that opioid antagonists exert more specific effects on ethanol consumption when administered at low doses (Herz, 1997). This author suggested that, given that the opioid system interacts with the mesencephalic dopamine, which mediates the reinforcement of food and water intake, it appears unlikely the opioid antagonists act on ethanol with absolute specificity. In fact, it has been found that administration of DAMGO, a mu opioid receptor agonist, in the nucleus accumbens augments the ingestion of saccharine, salt and ethanol, but not of water (Zhang & Kelley, 2002). These authors suggest that mu receptors regulate the ingestion of food throughout the evaluation of the hedonic value of the flavor. In the same vein, some results support the hypothesis that under the effects of an opioid antagonist the flavor of ethanol is perceived as less pleasurable, thereby decreasing the intake of the substance (Yeomans & Gray, 2002).

Prenatal exposure to ethanol and the opioid system

When it comes to prenatal exposure to ethanol, it has been shown that the opioid system plays a very important role in the reinforcing effects of the drug. Continuing with the last paragraph of the third chapter of this dissertation on prenatal ethanol exposure effects, Chotro and Arias (2003) began a line

of enquiry focused on prenatal preference conditioning with ethanol taste/odor as the CS but with the pharmacological effects of ethanol as the US. Some authors had previously demonstrated that the administration of opioid antagonists resulted in a decrease in ethanol consumption (Rohsenow et al., 2000; Yeomans & Phillips, 2002). Further, as explained before, it is known that the rat fetus on GD 17 already has a relatively mature opioid system and that this system participates in prenatal associative learning (Herlenius & Lagercrantz, 2001; Kehoe, 1988). Based on this background, and given that the pharmacological effects of ethanol are mediated by the endogenous opioid system, the authors considered the possibility of the involvement of the latter in the conditioned preference observed in the prenatal ethanol exposure effect. Therefore, using this hypothesis as a starting point, they began a series of experiments in which they administered opioid antagonists to interfere with the establishment of the association responsible for the conditioned preference. In the first experiment they gave a daily injection of an unspecific opioid receptor antagonist (naloxone) together with intragastric administration of ethanol (1 or 2 g/kg, a moderate to low dose) on GD 17, 18, 19 and 20. The pups were then tested for ethanol consumption two and four weeks after birth. The results demonstrated increased ethanol consumption on both test days for the group prenatally exposed to the drug only. The group exposed to the antagonist together with ethanol, however, showed the

same level of ethanol intake as the control group, i.e. the exposure to ethanol together with the antagonist blocked the increased intake of the drug. Similar results were found when they searched for changes in ethanol palatability after prenatal ethanol exposure (2g/kg) with or without the antagonist. Infant rats prenatally exposed to only ethanol showed a higher rate of appetitive responding for the ethanol flavor while subjects given ethanol plus opioid receptor antagonist showed a decrease in appetitive responding to ethanol (Arias & Chotro, 2005b). On the basis of these results, the authors concluded that the increased acceptance of ethanol after prenatal exposure could be explained by a conditioned preference learned by the fetus, associating the chemosensory properties of ethanol with a positive reinforcer that stimulates the opioid system. It is worth noting that this prenatal learning appears to be strong enough to be retained for 2-4 weeks after birth and to interfere with postnatal learning by reducing aversions and potentiating preferences when ethanol is involved (Arias & Chotro, 2006; Chotro, Arias, & Spear, 2009).

Other authors have found evidence consistent with the suggestion that the amniotic fluid contains endogenous opioids, and that the activation of these opioids may be responsible for the reinforcing effects that take place in uterus (Korthank & Robinson, 1998). It has been found that in a wide variety of mammals (including rats and humans), the amniotic fluid and other birth-related tissues, such as the placenta, contain a substance known as "placental opioid-enhancing

factor" (POEF) (Kristal, 1991). When ingested, this substance (which has not yet been chemically identified) activates the opioid system and potentiates the antinociceptive effects of opiates, although by itself it apparently does not induce any analgesic effect (Abbott et al., 1991; Kristal, 1991; Kristal, Thompson, Abbott, Dipirro, Ferguson, & Doerr, 1990). Another interesting connection between amniotic fluid and the activation of the endogenous opioid system is that described by Korthank and Robinson (1998). These authors found that the rat's amniotic fluid during the last 2 days of gestation (GD 20–21) contains substances that activate the kappa opioid receptor system of the fetus and the neonate (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010). This effect, known as the kappa-inducing factor (KIF), has been found to induce the stimulation of the kappa opioid receptor subsystem when it enters the subject's mouth (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010; Robinson & Méndez-Gallardo, 2010) and the authors suggest that the activation of KIF may be the agent that mediates prenatal conditioned preferences to flavors, including the effects of an increased liking for alcohol observed in our studies.

At this point, Díaz-Cenzano and colleagues (2010; 2014) started a series of experiments trying to elucidate the identity of the reinforcer by examining the role of the two main candidates: alcohol itself and the opioid receptors present in the amniotic fluid. To test the "KIF hypothesis", as KIF is present in the amniotic fluid starting on GD 20, while ethanol

activates the opioid system on any day, the authors exposed one group of rats on GD 17 and 18 and another group on days 19 and 20. They hypothesized that if increased ethanol acceptance was observed after prenatal exposure only on days 19 and 20, the results would be consistent with the KIF hypothesis, i.e. that the reinforcer is a substance of the amniotic fluid. However, if the effect was observed to be independent of the period of exposure, this would support the idea that ethanol was the agent activating the endogenous opioid system. The results obtained in this experiment showed a prenatal ethanol exposure effect in infant and juvenile rats when exposure was conducted on GD 19–20, but not on the previous days, which supported the KIF hypothesis (Díaz-Cenzano & Chotro, 2010). However, using specific antagonists for kappa and mu opioid receptors, it was observed that the effect was mainly mediated by mu opioid receptors, giving support to the hypothesis of ethanol effect being the reinforcer (Díaz-Cenzano, et al 2014). In view of this apparent inconsistency of the results, the specific effects of the amniotic fluid were tested by administering substances with flavor but with no pharmacological properties, to the pregnant mother. Nevertheless, the administration of vanilla or anise on those days of gestation (19-20) on which KIF is supposed to be acting, did not induce increased intake or enhanced palatability of those flavors when tested in infancy, unlike the case with alcohol (Diaz-Cenzano, et al., 2014). On the basis of these results, the authors concluded that it was still unclear whether

the stimulation of the opioid receptor system was induced by ethanol or by one of the components of amniotic fluid (or both) since the administration of a kappa opioid receptor antagonist also influenced the palatability of ethanol. Moreover, the authors suggested that it may be possible to find increased palatability to non-pharmacological flavored substances such as anise or vanilla after prenatal exposure if pups were tested closer to birth instead of later, on PD14 (Díaz-Cenzano et al., 2014).

ACETALDEHYDE

General aspects

Acetaldehyde can be produced both biologically and artificially, and significant amounts of this molecule can be found in some foods, beverages, cigarette smoke, and automobile exhaust. It is a highly volatile liquid with an unpleasant and irritating odor at room temperature and pressure. It is extremely reactive and has a very fast plasmatic elimination (Correa et al., 2012). Acetaldehyde is an intermediate in lactic and alcoholic fermentations, and is also a product of soya bean and cereal grain fermentation (Feng, Larsen, and Schnurer, 2007). It is, for instance, detected in bread, instant tea and coffee, roasted coffee beans, milk, yogurt and cottage cheese, and in many non- alcoholic and alcoholic beverages (Miyake and Shibamoto, 1993; Drake, Lopetcharat and Drake, 2009; Clemente-Jimenez, Mingorance-Cazorla, Martinez-Rodriguez, Las Heras-Vazquez, and Rodriguez-Vico, 2005). As mentioned previously, acetaldehyde is also present in tobacco smoke, in some cases at the same concentration as nicotine (Hoffmann, Hoffmann, and El-Bayoumy, 2001).

However, given the topic of this thesis, this section will primarily focus on the acetaldehyde that is derived from the metabolization of alcohol. As explained previously in the chapter about ethanol, acetaldehyde is originated both

peripherally and centrally after ethanol consumption. In the periphery there are two oxidative systems that convert ethanol into acetaldehyde. The first and the main enzyme is ADH, which is present in the liver, and the second involves the cytochrome P450 2E1, which is believed to convert less than 3% of the total ethanol in acetaldehyde (Hipolito, Sánchez, Polache and Granero, 2007). As mentioned previously, there are three important facts that prompted researchers to discover 30 years ago that acetaldehyde was formed directly in the brain and not by the hepatic-ADH enzyme. The first fact was that ethanol easily crosses the blood brain barrier; the second one that the peripheral acetaldehyde crosses the blood brain barrier with difficulty due to the presence of ALDH, which rapidly converts the acetaldehyde into acetate (Deitrich, 1987; Eriksson and Sippel, 1977; Zimatkin, 1991); and finally, the failure to find ADH in the rat brain, whilst detecting acetaldehyde in the brain after ethanol consumption (Amit, Brown and Rockman, 1977). These three important forced researchers to consider the possibility that other systems are responsible for metabolism of ethanol into acetaldehyde, such as the catalase enzyme system. Cohen and colleagues (1980) were the first authors to suggest that acetaldehyde could be formed directly inside the brain by the action of the enzyme catalase. They found that the neonatal rat brain was using this system, since there was a general lack of ADH in their brain whilst they had a very high amount of catalases in several brain structures (Cohen, Sinet and Heikkila, 1980; Del

Maestro and McDonald, 1987). Since then, many studies have been published showing in vivo and in vitro demonstrations of the catalase activity in the brain (Aragon, Spivak and Amit, 1991; Aragon and Amit, 1992; Zimatkin and Lindros, 1996; Zimatkin, Liopo, and Deitrich, 1998). However, other studies on catalase inhibition have shown that part of the oxidation of ethanol is not affected after inhibiting catalases, and have suggested that some other enzymes must be involved in this process (Aragon, Rogan and Amit, 1992; Gill, Menez, Lucas and Deitrich, 1992; Hamby-Mason, Chen, Schenker, Perez, and Henderson, 1997; Zimatkin et al., 1998). This is the case of CYP2E1, which is present in the liver and also in the brain (Hipolito et al., 2007), and through pharmacological manipulations it has been found to reduce acetaldehyde accumulation in the rat brain following ethanol intake (Zimatkin, Pronko, Vasiliou, Gonzalez and Deitrich, 2006). Evidence on ethanol metabolism in the brain suggests that approximately 60% of brain ethanol metabolism is accounted for by catalase, 20% by CYP2E1 (Zimatkin et al., 2006), and the remainder by other sources yet to be described (Zimatkin et al., 1998; 2006).

Behavioral effects of Acetaldehyde

Based on the studies mentioned previously, some researchers have suggested that the behavioral effects of ethanol could be produced by acetaldehyde, and more

specifically by the central acetaldehyde produced mainly by the catalase system (Correa et al., 2012). Moreover, some authors have ventured that ethanol could be a pro-drug, which means that the pharmacological effects that it exerts (both aversive and reinforcing) are produced mainly by its first metabolite, and not directly by ethanol itself (Karahanian et al., 2011; Quertemont & Tambour, 2004).

Over the last 20 years the approach to this issue has involved manipulating the enzymatic production and degradation of acetaldehyde through pharmacological manipulations to determine their influence on the reinforcement of ethanol (Hipolito et al., 2007). In one of the first experiments on this topic, the activity of ALDH was blocked with the use of Cyanamide (inhibits the activity of ALDH), which resulted in an acetaldehyde accumulation and a decrease of ethanol consumption (Amit, Levitan and Lindros, 1976; Sinclair and Lindros, 1981). Similarly, in another study using cyanamide together with ethanol, the authors reported a decrease in locomotor activity (Spivak, Aragon and Amit, 1987). From these results the authors concluded that systemic ALDH was blocked producing an increase of acetaldehyde in blood and subsequently enhancing the aversive effects of this substance. In addition, it appears that animals or humans that suffer from the mutation of ALDH 2, and in turn have higher levels of acetaldehyde, are somehow protected from ethanol abuse due to the excessive aversive effects they suffer after ingesting ethanol (Wall, Thomasson, Schuckit and Ehlers, 1992;

Hahn et al., 2006; Chen, Peng, Tsao, Wang, Lu and Yin, 2009; Chao, 1995; Chen et al., 1999; Peng, Chen, Tsao, Wang and Yin, 2007; Rivera-Meza, Quintanilla, Tampier, Mura, Sapag and Israel, 2010). This is the main reason why the use of drugs inhibiting this enzyme (ALDH) such as Disulfiram, are used in the pharmacological therapy of alcoholism. However, more recent studies using animal models of alcoholism have demonstrated that the use of ALDH inhibitors is not completely effective since rats develop tolerance to Disulfiram (Tampier, Quintanilla and Israel, 2008). Other studies also show how it only reduces consumption to 50% while in naïve rats they found a reduction in intake of 90% (Ocaranza, Quintanilla, Tampier, Karahanian, Sapag and Israel, 2008; Garver, Ross, Tu, Cao, Zhou and Israel, 2000; Tampier et al., 2008).

In relation to the manipulation of ADH, it has to be taken into account that this enzyme is not present in the brain and that its inhibition only avoids peripheral acetaldehyde accumulation, but not the central acetaldehyde formation (Zimatkin et al., 2006). For instance, it has been demonstrated that the administration of 4-methylpyrazole (4MP, an ADH inhibitor) reduces the acetaldehyde blood levels (Jamal et al., 2007) and that this increases motor incoordination (Rydberg & Neri, 1972), but it does not affect locomotor activity or conditioned taste aversion (Spivak et al., 1987). Among those studies including manipulation of ADH, the use of the drugs D-penicillamine and L-cysteine have yielded interesting results. Both are thiol compounds that interact with acetaldehyde

non-enzimatically to form stable adducts, and have been demonstrated to reduce acetaldehyde blood levels after ethanol administration (Kera, Kiriya and Komura, 1985; Nagasawa, Elberling and DeMaster, 1980). Experiments using these sequestering agents have shown that voluntary drinking of ethanol is decreased and that when treatment with D-penicillamine is complete, rats return to their normal ethanol intake (Font, Aragon & Miquel, 2006). Moreover, L-cysteine reduces acquisition and maintenance of oral ethanol self-administration and reinstatement after ethanol extinction in rats (Peana et al., 2010a). Using an operant paradigm with rats, D-penicillamine was able to block the acquisition of operant ethanol self-administration, but not maintenance of the response (Peana, Porcheddu, Bennardini, Carta, Rosas, & Acquas, 2015). Results from another study showed that D-penicillamine was able to prevent ethanol relapse-like drinking (Martí-Prats, Zornoza, López-Moreno, Granero, & Polache, 2015). When it comes to the anxiolytic effects produced by administration of moderate doses of ethanol, it has been demonstrated that sequestering acetaldehyde with D-penicillamine blocks these anxiolytic effects (Correa, Manrique, Font, Escrig, & Aragon, 2008).

Another approach to the study of centrally generated acetaldehyde has been the manipulation of the catalases by the use of 3-amino-1,2,4-triazole (AT), sodium azide, lead acetate or using acatecolamic subjects. Following the vast literature measuring the effects of inhibiting and blocking

those enzymes involved in the metabolization of ethanol in blood, in recent years this has gathered importance in the investigations of the behavioral significance of centrally generated acetaldehyde (Correa et al., 2012). Those studies described previously, in the General Aspects section, demonstrate that catalases are responsible for the highest proportion of central ethanol metabolism (Aragon et al., 1992; Zimatkin et al., 2006) while CYP2E1 also contributes to some degree to this process (Zimatkin et al., 2006; Hipolito et al., 2007; Correa et al., 2009a). There are several studies demonstrating, both in humans and rats, a positive correlation between brain catalase activity and ethanol consumption (Gill, Ait, & Smith, 1996; Aragon, Sternklar, & Amit, 1985; Amit & Aragon, 1988; Koechling, Amit, & Negrete, 1995; Koechling and Amit, 1992; Amit, Smith, & Weiss, 1999; Amit and Aragon, 1993). Moreover, the inhibition of catalases results in an attenuation of acquisition (Rotzinger, Smith, & Amit, 1994) and maintenance (Amit and Aragon, 1992a; Koechling and Amit, 1994; Tampier, Quintanilla, & Mardones, 1994) of voluntary ethanol intake in rats and mice (Aragon and Amit, 1992a; Koechling and Amit, 1994). In addition, with the administration of AT, ethanol-induced conditioned place preference was impaired in a dose-dependent manner (Ledesma et al, 2013), and operant responses to ethanol were also reduced (Peana et al., 2015). The use of sodium azide and AT to inhibit catalase activity has also demonstrated to lower the anxiolytic effects of ethanol, measured by performance in an elevated plus maze

and in a dark/light box. In contrast, the administration of lead acetate, which potentiates catalase activity, has been demonstrated to have opposing effects in the same study (Correa et al., 2008).

The last approach on this topic has been to administer acetaldehyde directly into the brain. Although a very volatile compound, it is possible to administer acetaldehyde through different routes to assess the behavioral effects that it exerts (Correa et al., 2012). In general, ethanol and acetaldehyde show very similar effects after being administered to the organism, activating motor activity and producing Pavlovian aversive and appetitive learning when associated with a conditioned stimulus (Correa et al., 2012). Acetaldehyde can also act as a reinforcer in operant conditioning procedures. Ethanol non-dependent rats trained in operant chambers for 11 days were able to show self-administration of 2% or 5% of acetaldehyde directly into the brain (Amit et al., 1977; Brown, Amit, & Rockman, 1979; Brown, Amit, & Smith, 1980). The direct administration of acetaldehyde into the brain has been described to induce the same or even stronger anxiolytic effects when compared with ethanol, by increasing exploration of the central area of an open field (Correa, Arizzi, Betz, Mingote, & Salamone, 2003c) and increasing frequency and time into the open arms of an elevated plus-maze (Correa, Salamone, & Aragon, 2005a).

Taken together, the results of studies employing pharmacological manipulations of enzymes and metabolites derived from ethanol metabolism, have highlighted the importance of acetaldehyde as the agent responsible for both the aversive (peripheral production) and appetitive (central production) reinforcing effects of ethanol. It appears beyond doubt that acetaldehyde plays a central role in the various effects of ethanol, such as anxiolytic effects, increased or reduced intake, and locomotor activation. Further these findings may help us to understand the mechanisms underlying the reinforcing effects of this drug.

Acetaldehyde in infant rats

There are a small number of studies that analyze the behavioral effects of acetaldehyde in early ontogeny. In one of these studies with neonatal rats, researchers used the Pavlovian appetitive conditioning paradigm and administered ethanol or acetaldehyde (US) to the pups followed by exposure to an olfactory cue (CS). They found that with both drugs the CS was recognized and appetitively conditioned. In a second experiment, however, they used D-penicillamine to sequester the acetaldehyde from the system and they found an inhibition in the appetitive conditioned response (March, Abate, Spear, & Molina, 2012). In addition to this evidence, infant rats of 14 days old developed a tactile conditioned

preference to a CS previously paired with ethanol, and when D-penicillamine was administered together with ethanol the motivational and locomotive effects were blocked (Pautassi, Nizhnikov, Fabio, & Spear, 2011). To our knowledge, there are no studies examining the role of acetaldehyde when the ethanol exposure takes place during gestation.

However, in relation to this topic, it is important to note that the neonatal period is considered a sensitive period in which rat pups acquire appetitive conditioning more readily than aversions, as mentioned before in reference to the results from Sullivan and colleagues (2003; 1994). With respect to ethanol, Arias and Chotro (2006) confirmed the findings of Sullivan and found that ethanol intoxication on postnatal days 7 and 8 increased ethanol intake and enhanced ethanol's palatability when tested 3 days later, but the intoxication on days 10 and 11 decreased ethanol intake and increased aversive responses to the drug. It was also found that the opioid system plays a critical underlying role in the effects observed during this sensitive period (Roth & Sullivan, 2001; 2003). In particular, the administration of an opioid antagonist during this period disrupts the acquisition and consolidation of odor preference learning. The critical role of the opioid system is also supported by data showing that by the end of this period this neurochemical system suffers several changes, particularly in brain areas known to mediate reward and learning (Roth & Sullivan, 2003).

During this sensitive period, however, the opioid system is not the only critical system with high activation in the brain. Catalases also show heightened activation, beginning during the fetal period and finishing around postnatal day 20 (Zimatkin, 2013; Zimatkin and Lis, 1990) and this directly correlates with the period in which rat pups show an acceptance for intoxicating doses of ethanol (Hamby-Mason et al., 1997; March, Abate, & Molina, 2013). As the catalases are the enzymes responsible for converting ethanol into acetaldehyde in the brain, it is suggested that the reinforcing effects of central acetaldehyde are related to the high activity of these enzymes during this developmental stage (March et al., 2013). In addition, it is necessary to consider that the capacity of the fetus to clear acetaldehyde by means of ALDH is limited due to the null or low activity of this enzyme early in ontogeny (Zimatkin, 2013). In fact, it was found that the activity of ALDH in the rat brain was at 45-70 % on PD 10 when compared with an adult, and approaches the definitive level on PD 20 (Zimatkin and Lis, 1990). Thus, it is suggested that both the higher activity of catalase and the low activity of liver-ALDH could be responsible for the reinforcing effects produced by acetaldehyde (Zimatkin, 2013).

Even though there are few studies analyzing the pharmacological and behavioral effects of acetaldehyde in neonatal subjects, the data appear to support the sensitive period theory previously described.

To summarize all the findings described in this chapter, it is clear that acetaldehyde is an important metabolite as it has been demonstrated to be reinforcing. Therefore, when researching the effects of ethanol it is important to consider this metabolite together with other hypothesis such as the one focused on the reinforcing effects mediated by the opioid system. Various experiments have been conducted to clarify the role of peripheral and central acetaldehyde using different inhibitors and sequestering agents. The results show that in general terms, the central acetaldehyde that originates from the catalase system in the brain, is responsible for the positive effects of ethanol, whilst the peripheral acetaldehyde is responsible for the aversive effects. In the case of rat fetuses and neonates, this effect has also been replicated since they have insufficient maturity to acquire peripheral acetaldehyde whilst they have an increased level of catalases able to generate central acetaldehyde. Moreover, with respect to the reinforcing effects, we have reviewed the theories about the sensitive period related to the opioid system, but also added the possibility of a period system resulting from the heightened action of catalases until PD 10. To our knowledge there have been no studies carried out to search for the role of acetaldehyde in the prenatal ethanol exposure effect. Therefore, and given the suggested importance of acetaldehyde on the basis of previous work, some of the objectives and hypotheses of this dissertation are guided by this finding.

OBJECTIVES & HYPOTHESIS



OBJECTIVES

General aim:

The general aim of the research presented in this thesis was to further investigate the identity of the reinforcer that may be acting during ethanol exposure on the last days of gestation, and by which rat fetuses seem to learn a conditioned preference for ethanol flavor.

Specific objectives:

The first objective was to test the possible role of the amniotic fluid (and its component KIF) in the induction of a conditioned preference to a flavor experienced during the last days of gestation. Therefore, the effect of prenatal administration of a non-ethanol flavor (vanilla) was assessed at different postnatal stages of early development, using different evaluation techniques adequate to the pups' sensorial and motor capacities.

Considering the postulated differential effects of the amniotic fluid and ethanol on the kappa and mu opioid receptor systems, the second objective was to study the implication of the mentioned receptor systems during prenatal exposure to ethanol and to a non-ethanol flavor (vanilla) on the prenatal acquisition of a learned preference to them. Therefore the neonatal attraction for those flavors was

tested as a function of their prenatal exposure together with kappa or mu opioid receptor antagonists.

The third objective was to analyze the role played by the first metabolite of ethanol, acetaldehyde, on the appetitive response to ethanol's flavor observed after its prenatal exposure. To prove this, responses to ethanol flavor were tested at different postnatal ages with different procedures, in subjects exposed prenatally to ethanol together with an acetaldehyde-sequestering agent.

HYPOTHESES

The general working hypothesis of this dissertation is that the main reinforcer involved in the learned preference acquired in utero, as a consequence of prenatal ethanol exposure, is related to the pharmacological effects of the drug. The specific hypotheses are in correspondence with the three specific objectives proposed.

On the first place, although the amniotic fluid and its component KIF have certain effects on the opioid system, we expect that contrarily to what it is observed with ethanol, the prenatal exposure to a non-ethanol flavor such as vanilla will not induce an increased acceptance of this flavor, at least when tested as late as two weeks after birth. However, when assessing the pup's response closer to birth, a differential response to vanilla may be observed, in accordance with the results of previous studies in humans and animals.

Secondly, if KIF from the amniotic fluid is the responsible for the increased acceptance for vanilla after its prenatal exposure, the simultaneous administration of a kappa- (but not mu-) opioid receptor antagonist will prevent the observation of that effect. On the other hand, with prenatal exposure to ethanol, we expect to find an increased attractiveness to ethanol odor after its prenatal exposure, which will be mainly affected by blocking the mu-opioid receptor system.

Thirdly, if acetaldehyde plays an important role in the reinforcing effects of prenatal ethanol, we expect to prevent this reinforcement when eliminating the metabolite, using an acetaldehyde-sequestering agent during the prenatal ethanol exposure.

GENERAL METHODS



Subjects

For all experiments presented in this thesis a total number of 833 Sprague-Dawley rat pups were used, derived from 140 litters (the specific number used will be shown in the corresponding experiment). Rats were born and reared in a temperature-controlled vivarium at the University of the Basque Country (Spain). The colony room was maintained on a 12-h light/12-h dark illumination cycle, with light onset at 8:00 am, and with an appropriate temperature (21-23 °C) and humidity (50-60 %). Adult female rats were time-mated to provide subjects for this study, and the presence of sperm in vaginal smears was considered as gestational day 0 (GD 0). Pregnant females were housed in pairs in standard maternity cages with continuous access to food (Panlab, Spain, maternity formula) and filtered tap water, and remained undisturbed until the initiation of the different treatments on GD 17. At the end of the procedure on GD 20, dams were housed individually and remained undisturbed for parturition (GD 22). Maternity cages were checked daily for birth, from 9:00 to 14:00, and if positive, these were considered as postnatal day 0 (PD 0). European regulations for the care and treatment of experimental animals were followed, and procedures were controlled and approved by the "Ethics and Animal Care Committee" at the University of the Basque Country UPV/EHU (CEBA) and the Diputación Foral de

Gipuzkoa, Spain, in compliance with the European Communities Council Directive (86/609/EEC).

Procedures

Prenatal Treatment

Prenatal treatments for the experiments of this study were carried out from GD 17 to GD 20. At the beginning of the procedure, dams were removed from their home cages, marked on the tail for identification, and weighed. After being weighed, all the rats received an intragastric administration of the substance corresponding to each treatment and experiment (ethanol, vanilla, or water). The intragastric administration was performed using a 15-cm length of polyethylene tubing (PE-50 Clay Adams, Parsippany, NJ) attached to a 10 ml syringe with a 24-gauge needle. The tubing was gently inserted through the mouth and slowly pushed into the stomach. The entire procedure took around 15 sec per rat.

In experiments 4, 5, 6, and 7 pregnant rats received a drug (i.e. an opioid receptor antagonist, D-penicillamine, or saline) together with the substance administered intragastrically. For the administration of the drugs, a subcutaneous injection (in the area of the nape) was given to each rat 30 min before the intragastric administration, on every prenatal administration day (except for the drug Nor-

Binaltorphimine in Experiment 4 which was administered only on GD 17 and 19 due to its long lasting effect).

After the corresponding treatment, dams were placed back in their home-cages until PD 20, when these same procedures were repeated. All treatments began at 8:00 am on every treatment day. After the prenatal treatment on GD 20, dams were housed individually and remained undisturbed for parturition.

Postnatal Tests

Within the seven experiments presented here, the postnatal behavior of the pups was evaluated using four different techniques: intake test, taste reactivity test (TRT), an odor induced crawling locomotion test, and operant conditioning.

Intake test:

A female and a male from each litter were evaluated on two intake tests, one of water and one of the corresponding substance, both tests being separated by a one-hour interval. The exception was in Experiment 1 in which only one intake test was conducted with each pup, either water or the flavored substance (vanilla). At the commencement of the procedure, pups were separated from their mother, marked on the tail for

identification, and cannulated. For the intraoral cannulation we used a procedure already described in previous studies (Chotro & Arias, 2007). In brief, cannulas were made from 7-cm sections of PE-10 polyethylene tubing with an internal diameter of 0.28 mm (Clay Adams, Intramedic). One end of the section was heated to form a small flange. A thin wire attached to the non-flanged end of the cannula was placed on the internal surface of the pup's cheek. The wire was then pushed through the oral mucosa until the flanged end of the cannula was positioned over the internal surface of the cheek while the remainder of the cannula exited from the oral cavity. The entire procedure took no more than 10 seconds per pup and induced minimal stress (Spear, Specht, Kirstein, & Kuhn, 1989). These cannulas were then connected to the syringes using a PE-50 polyethylene tube containing the substances placed in an automated pump (KDS Scientific). This pump was scheduled to administer the fluids at a rate of 0.1 ml/min per infusion for 15 minutes (i.e. 1.5 ml of the given substance) with a continuous flow. Following the cannulation procedure, the subjects were grouped according to litter in heated holding chambers (15 x 8 x 15 cm) for one hour before the test. A few minutes before the test, the pups' bladders were voided by gently brushing the anogenital area, and body weights were then registered. The pups were then tested in individual clear plastic chambers (8 x 8 x 25 cm).

In all cases, pups could either consume or reject the infused fluid during the tests. At the end, postinfusion weights

were registered and pups were returned to the holding cages. Intake of the intraorally administered fluids was calculated considering pre- and post-infusion body weights and expressed as a percentage of body weight gained (% bwg). To finish the procedure, cannulas were removed and pups were returned to their home cages.

Taste reactivity test (TRT):

A female and a male from each litter were separated from their mothers, marked for identification, intraorally cannulated and placed in holding chambers (15 x 8 x 15 cm) maintained at 28-30 °C with heating pads. Pups were cannulated intraorally using the same procedure previously explained for the intake test. The evaluation was conducted in a trapezoid-shaped chamber with a front wall (29 cm wide) made of clear glass, and the remaining walls (back 18 cm, sides 11.5 cm, 12.5 cm high) and floor were mirrored, so as to allow observation of the pups' orofacial expression and body movements in any position. The chamber was divided into two equal sections, so as to allow pups to be evaluated at the same time, one in each section of the chamber. The pups remained in the test chamber for an adaptation period of 10 min before the start of the test. The test was 7 min long and consisted of the delivery of 2 intraoral infusions of water followed by 5 infusions of the flavor. Intraoral infusions were performed using an automated pump (KDS Scientific) scheduled to

administer the fluids at a rate of 0.1 ml/min per infusion. This pump was connected to the intraoral cannula of each pup. Infusions were 15-seconds long, separated by intervals of 45 seconds. Thus, pups received one infusion per minute, and the volume of each infusion was 0.025 ml. During the entire test the pups were videotaped from the front glass wall for subsequent analysis of their behavior. Based on previous studies from our laboratory with rat pups (Diaz-Cenzano, Gaztañaga & Chotro, 2014), we considered two categories of reactions - hedonic and aversive. Within the hedonic (or appetitive) reactions we included mouthing (with and without tongue protrusion) and paw licking. Within the aversive category the behaviors included were gaping, head shaking, forelimb flailing, paw treading, chin rubbing, and wall climbing. In accordance with the nature of the reaction categories, on each trial we measured the duration (seconds) of hedonic reactions and the frequency (number) of aversive reactions. To finish the procedure, cannulas were removed and pups were returned to their home cages.

Odor induced crawling locomotion test:

Given that crawling is a very unique behavior that is only displayed shortly after birth, this technique was used as a measure of performance exclusively in PD1 neonates. This "odor-induced crawling" test was adapted from the procedure described by Méndez-Gallardo & Robinson (2014). A female

and a male from each litter were tested with one odor (vanilla, ethanol or water) and the attractiveness to this odor was measured by the following procedure. A cloth strip was placed over a heating pad on a table, creating a warm and solid base on which the pups could crawl. To one side of this runway a ruler was glued in order to easily measure the distance travelled by the pups. All the movements of the neonates were recorded by a video camera placed above the runway. To expose pups to the odors a small amount (0.3 ml) of the testing solution was placed in a 1.5 ml graduated microcentrifuge tube containing a small ball of cotton at the bottom. At the beginning of the session, pups from each litter were removed from their home cage, marked for identification, and then placed in groups according to litter in an incubator at 35 °C during a period of 30 minutes to acclimate. The pups were then placed into a holding chamber at 27 °C for a further 30 minutes. After this period, subjects were tested one by one, placing them unrestrained in a prone position at the beginning of the runway, with the nostrils aligned with the starting line. The tube containing the odor was positioned by the experimenter immediately in front of the pup's snout, and maintained continuously for a period of 2 minutes. If the subject travelled the complete runway (80 cm) within 2 minutes, the testing session for that subject was completed. Otherwise, the session ended after 2 minutes of exposure to the odor. As mentioned in the study by Méndez-Gallardo & Robinson (2014), pups can easily lose contact with the tube

and/or stop moving and subsequently fall asleep. In the event of the neonate losing contact with the tube, this was replaced over the pup's snout immediately, and to prevent the neonates from falling asleep the tube was removed and placed again over the pup's snout after 30 seconds of inactivity. While the pups were responding to the odor by crawling and pushing the tube, the investigator moved the tube forward, taking care not to interrupt the behavior of the subject.

The measure of this test was the distance in cm travelled on the runway by each subject within the 2 minutes test session. This measure was registered immediately after testing each subject by a researcher blind to the prenatal treatment and to the testing condition of the pups. However, as mentioned before, all tests were digitally recorded and video-files were kept to provide evidence of the experimental results, and to allow for reviewing scores in case of experimenter error.

Operant conditioning:

This technique was used to test the response to the substances at two different postnatal stages: PD 5 and PDs14-17. The procedures were therefore adapted to each age accordingly.

Operant test on PD 5: for this test, the procedure and apparatus were similar to those described by Arias et al. (2007).

After being cannulated using the same procedure explained before, pups were maintained undisturbed for 3 hours at 30 °C in an incubator. After the separation time, their bladders were voided by gently brushing the anogenital area, at which point their body weights were registered. The subjects were then placed in the testing chamber and their intraoral cannulas were connected to an infusion pump through a section of PE-50 polyethylene tubing attached to the needle fitted into the tip of the syringe of the infusion pump. The rat pups were then placed in a semi-supine position over a "holding seat" constructed using the internal cotton surface of a respirator mask (3 M Particulate Respirator 8576), while this seat was positioned over a metal support box. The angle between the pup's body and the surface of the box was equivalent to 40°, which allowed the pup to rest its rear limbs over the filter of the respirator mask. Each pup was strapped and buckled into a spandex "vest" with a "v"-shaped neck designed to avoid restriction of head movements. Two holes (0.5 cm in diameter) in this vest allowed the pup's forelimbs to be free. The vest produced no apparent discomfort or major restriction of behavior. An articulated iron stand equipped with alligator clips allowed positioning of a touch sensitive bronze sensor (4 cm long and 0.5 cm wide) 1.5 cm away from the pup's mouth and perpendicular to the base of the holding seat. The tip of this sensor was kept equidistant from each forepaw. Physical contact with the sensor activated an infusion pump (Kashinsky-Rozboril, Model 5/2000, Binghamton, NY) equipped

with a 2-ml micrometer syringe (Gilmont Instruments; Barrington, IL) filled with a specific solution. The sensor was connected to a single channel charge-transfer sensor chip (Model E11x Evaluation Board; Quantum Research Group, Pittsburgh, PA), which in turn controlled the infusion pump. The pump was set to deliver 1 ul of solution whenever the sensor was activated (the schedule of reinforcement was set as a fixed ratio 1). The sensor chip was also connected to a device (Simple Logger II, Model L404, AEMC Instruments, USA; sensitivity: 1 response/0.01 s), which registered, in real time, the number of sensor contacts displayed by the animals. Therefore, the dependent variable was the number of touches registered by this device. The duration of the test was 15 minutes, and in the case of Experiment 7 a six-minute extinction session was added.

Operant training and test on PD 14-17: the procedures and apparatus employed here were similar to those described in previous studies (Miranda-Morales, et al., 2012; Pautassi, et al., 2008). Operant training with older animals was conducted on various days: PD 14, 16, 17 and 18. The first 3 days were training sessions while the last day was considered as the test. On each day the pups were removed from their maternal cages, cannulated and placed in pairs in a holding cage lined with pine shavings and warmed to approximately $35\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$. Two hours later, operant training took place. First, pups bladders were voided before their weights were recorded. Custom-made operant-conditioning chambers (12 cm \times 12 cm

× 15 cm) were employed. One lateral wall of the chamber contained a hole (diameter: 1 cm); behind which was an evaluation board equipped with a circular touch-sensitive sensor (Model E11X Evaluation Board; Quantum Research Group, Pittsburgh, PA). The target behavior under training was nose-poke. In particular, each time the snout of an experimental subject touched the sensitive sensor a signal went on and activated an infusion pump. A 50-cm section of PE-50 polyethylene tubing was connected to the end of the oral cannula of the animal and to a 2-ml micrometer Gilmont Syringe (Gilmont Instruments, Barrington, IL) mounted on a rotary syringe-pump (Kashinsky-Rozboril, Model 5/2000, Binghamton, NY). The pump was set to deliver a liquid at the rate of 5 μ l/s, directly into the oral cavity of the animal (the schedule of reinforcement was set as a fixed ratio 1). Nose-poking frequency was recorded during each operant session (Simple Logger II, Model L404, AEMC Instruments, USA; sensitivity: 1 response/0.01 s). Immediately after each training session, the weight of each pup was recorded again to measure intake, and pups were returned to their home cages.

For both tests, pups were evaluated in pairs, one being the paired subject (P) and the other the yoked subject (Y). Whenever a P subject touched the sensor, a 1 μ l pulse of solution was delivered into its mouth as well as into the mouth of the corresponding Y control subject. Physical contact between Y subjects and the sensor was registered, but did not result in activation of the pump (no reinforcement). At the

beginning of the test, all pups received 2 priming pulses of the solution (60 and 120 s). Each priming pulse was equivalent to 1 ml, and these pulses were administered independently of the motor activity rates of the subjects. This allowed us to familiarize subjects with the reinforcer whilst minimally stimulating head and body movements.

Substances employed

On prenatal treatment:

In all of the experiments reported in this thesis three different substances were administered intragastrically to the pregnant dams: vanilla, ethanol, and water. The vanilla flavored solution used for prenatal administration consisted of 500 % w/v of vanillin (Sigma Aldrich) in filtered tap water, and the administered volume was equivalent to 0.01 ml/g of body weight. The vanillin concentration was selected from a study conducted in this same laboratory, which provides evidence that this dose can be detected in amniotic fluid (Diaz-Cenzano et al., 2014). The ethanol dose administered was 2 g/kg and resulted from the administration of a volume equivalent to 0.015 ml/g of a 16.8 % v/v ethanol solution in filtered tap water. This ethanol dose has been shown to reach the amniotic fluid and has been consistently found to be effective in generating the prenatal ethanol exposure effect, i.e. increased acceptance of

ethanol. Finally, control dams received a similar volume of filtered tap water.

As explained above, in some of the experiments (4-7) pregnant rats received a subcutaneous injection of one of the following drugs: the kappa (κ) opioid receptor antagonist, Norbinaltorphimine (NBI, 8 mg/kg), and the mu (μ) opioid receptor antagonist Naloxonazine (NXZ, 10 mg/kg). Vehicle for Norbinaltorphimine was an isotonic saline solution (0.9 % of NaCl), while for Naloxonazine it was a saline solution containing acetic acid (0.1 %). The antagonist doses were selected from previous studies (Arias, Molina, & Spear, 2010; Mitchell, Liang, & Fields, 2005; Wee, Orio, Ghirmai, Cashman, & Koob, 2009). With respect to D-penicillamine (DP), the dams received 150 mg/kg of this drug, injected subcutaneously. The control treatment for all these drugs was a subcutaneous injection of a similar volume of isotonic saline solution (0.9 % of NaCl).

On postnatal tests:

Intake test and TRT: In addition to the response to water, in these two tests the response to intraoral vanilla and ethanol was measured. For the vanilla flavor a 50 mg % solution of vanillin (Sigma Aldrich) in filtered water was employed, and for ethanol a 6 % v/v solution of 190% proof ethanol in filtered water was used.

Odor-induced crawling locomotion test: In all of the experiments employing this technique, the following three odors were used on the test : water, vanilla and ethanol. The vanilla odor consisted of a 50 % w/v of vanillin in filtered tap water and the ethanol odor of 6 % ethanol in filtered tap water. In the three cases 0.30 ml of each solution was placed in the cotton of the microtube.

Operant conditioning: On the vanilla operant test pups received intraoral infusions of either a vanilla solution (50 % w/v of vanillin) mixed with saccharin (0.5 mg/l; Sigma Aldrich) in filtered tap water, or vehicle (filtered tap water with 0.5 mg/l saccharin). Saccharin was added to the vanilla solution in the second experiment based on the results of a non-published pilot study from the same laboratory. In particular, we found that pups from PD 14 would show minimal responses on the operant task, but that responding could be increased by the addition of saccharin to the solutions. For the ethanol operant test, pups received 6 % v/v of ethanol or filtered tap water mixed again with saccharine (0.5 mg/l) as the reinforcer.

Data analysis

The experimental designed used for each experiment will be described in the corresponding section of each experiment. In general, data are analyzed using factorial or mixed ANOVAs and main effects and interactions are explored

with Duncan's post-hoc tests. The alpha level was set a priori at $p < 0.05$ for all analyses.

In order to avoid overrepresentation of a litter in each group, in all experiments only one pup per litter entered into each of the defined groups, and was tested in only one of the tests (either TRT, intake test, operant technique, or crawling). For all experiments the Sex variable was included in a pre-analysis and as data demonstrated that it was not significant in any of the experiments, this factor was excluded from the analyses and results presented in this thesis.

In the case of the hedonic and aversive reactions from the TRT, they were analyzed separately with mixed ANOVAs including Prenatal treatment as the between-subject variables, while Trial (7 trials: 2 infusions of water and 5 of the given substance) was included as a within-subject variable. Data from intake tests were analyzed with a mixed ANOVA including Prenatal treatment as the between-subject variable, and Test (water and flavor intake tests) as the within-subjects factor, with % bwg as the dependent variable. In the case of crawling, factorial ANOVAs were conducted with Prenatal treatment and Testing substance as independent variables, and the distance travelled as a dependent variable. All interactions were explored with follow-up ANOVAs for each level of the within-subject variable, whilst factorial interactions were analyzed with Duncan's post hoc tests.

EXPERIMENTS & RESULTS



EXPERIMENT 1

In this first experiment our aim was to test whether prenatal exposure to a flavored substance with no pharmacological effect would subsequently allow subjects to recognize and consume more of this substance than subjects given no prenatal exposure. As mentioned in the introduction, this aim was also assessed in previous studies carried out in this laboratory, but using different techniques or subjects of different ages. For instance, in a study by Díaz-Cenzano et al., (2014) subjects prenatally exposed to vanilla or anise, tested on PD 14 with intake tests and TRT, did not display differential responses to these flavors. As the authors explained, it is possible that the lack of a differential response that is otherwise usually observed with prenatal ethanol may be due to the relatively late age of evaluation. It is possible that the memories originating in utero after experiencing these non-ethanol substances were not strong enough to be retained until PD 14. Therefore, in this experiment we conducted the evaluation closer to birth, on PD 5 as well as on PD 14. The specific hypothesis of this experiment is that the prenatal exposure to vanilla will induce increased intake of vanilla detected on PD 5. In addition, the re-exposure to vanilla on the PD 5 test will serve as a reminder that will facilitate recognition and consumption of this substance on PD 14.

Specific Procedures

In this experiment the responses of the offspring were evaluated by an intake test on PD 5 (water or vanilla) and PD 14 (vanilla). As explained before, in this experiment pups were tested with only one substance on each test: either water or vanilla on PD 5. On PD 5, six pups from each litter were separated from the mother, identified and cannulated, but only four were evaluated: two (a female and a male) on a water intake test and other two (a female and a male) on a vanilla intake test. The two pups that remained after cannulation stayed in the holding cage and did not receive any substance (un-treated group, UT). On PD 14 all six pups from each litter were tested on a vanilla intake test.

Experimental design

For this experiment 50 subjects from 9 litters were randomly distributed into 6 groups defined by: Prenatal treatment (vanilla or water), and the Test substance on PD 5 (vanilla, water, UT) (see table 2).

Prenatal Treatment	Test on PD5	Test on PD 14
Water	Water	Vanilla
	Vanilla	Vanilla
	UT	Vanilla
Vanilla	Water	Vanilla
	Vanilla	Vanilla
	UT	Vanilla

Table 2: Experimental design of Experiment 1. UT: untreated group

However, data analyses were conducted separately for both ages. For the PD 5 test, data were analyzed by a factorial ANOVA (2 x 2) with the independent variables Prenatal treatment (water or vanilla) and Test substance (water or vanilla), and with Intake (%BWG) as the dependent measure. Logically, group untreated was not included in the PD 5 test analysis, since no data were collected at that age. Data from PD 14 were analyzed by a factorial ANOVA (2 x 3), with Prenatal treatment (water or vanilla) and the Test substance on PD 5 (water, vanilla, or UT), as independent factors, and Intake (%BWG) as the dependent variable.

Results

The factorial ANOVA with data from the intake test on PD 5 yielded a significant effect of Test substance $F(1,29) = 8.48$, $p < 0.01$, with all groups consuming significantly more vanilla than water, independent of the Prenatal treatment (see Figure 1).

The results of the ANOVA conducted on the intake test data on PD 14 indicated no significant effect of any variable, i.e. all subjects consumed similar amounts of vanilla independent of the Prenatal treatment and the Testing substance experienced on the intake test on PD 5 (see Figure 2).

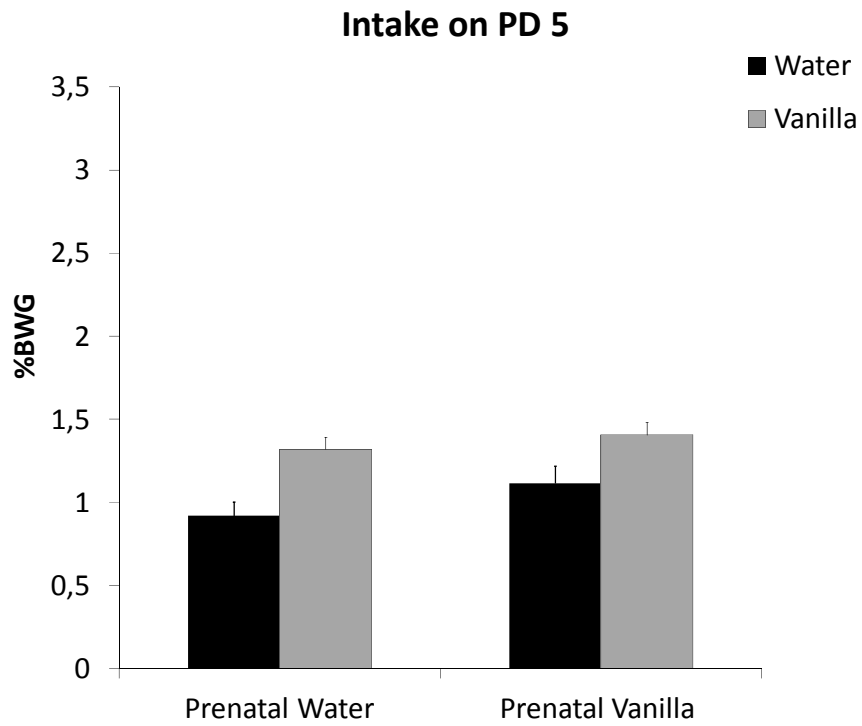


Figure 1. Mean (+ - SEM) water and vanilla intake on PD 5 as a function of the prenatal treatment (water or vanilla).

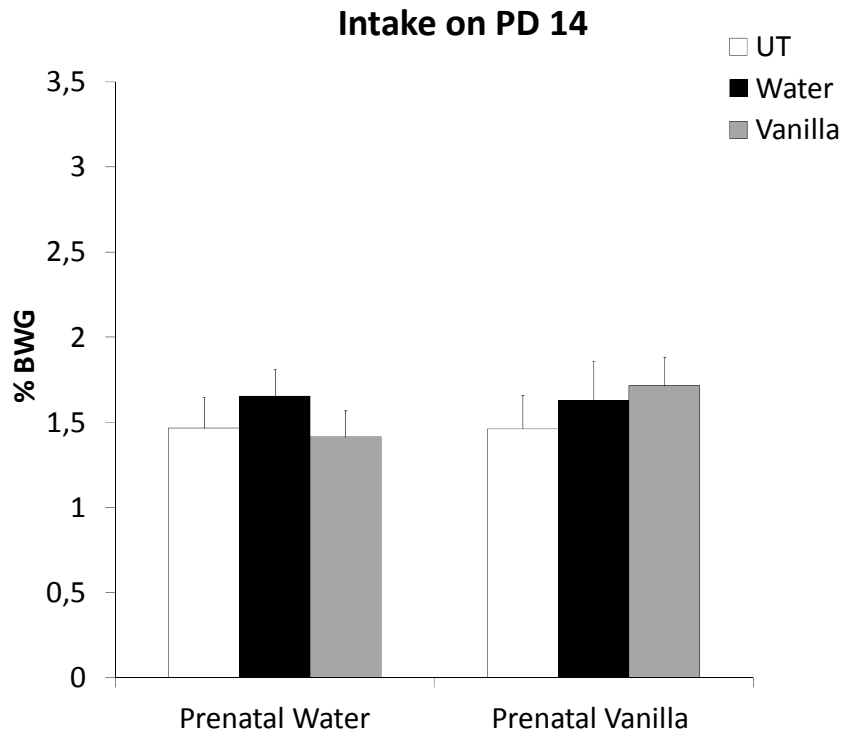


Figure 2. Mean (+ - SEM) water and vanilla intake on PD 14 as a function of the prenatal treatment (water or vanilla). UT: untreated group.

The results of the experiment show that evaluating pups earlier and giving a reminder before PD 14 did not generate the expected increase in the acceptance of vanilla in pups prenatally exposed to the substance. However, one might suppose that testing pups earlier than PD 5 could still allow the observation of the subjects' recognition of the substance experienced in utero. Moreover, it is possible that the intake test procedure is not accurate enough to test

acceptance of flavors at this early age, considering their immaturity for controlling ingestion in a passive intraoral infusion situation, instead of suckling. When tested at PD 14, however, previous studies have demonstrated that the intake technique is sensitive enough to measure the increased acceptance of flavors. Therefore, the null results obtained using subjects at this age, even in those cases in which a reminder was given on PD 5, confirm those results reported in previous studies (Díaz-Cenzano et al., 2014). On the basis of these considerations, in the subsequent experiments we decided to test our working hypothesis by using different techniques more appropriate to the early ontogenetic stage of the pups and/or using pups at an earlier age.

EXPERIMENT 2

The second experiment employed the same design as that described in the previous one except that the subjects' response to the flavored substance was assessed on PD 5 and PD 14, in an operant conditioning task. The decision to choose this test procedure was based on previous pilot studies in which the intake test used on PD14 did not generate reliable results when applied on PD5. However, the operant conditioning procedure developed by Arias, Spear, Molina, and Molina, (2007) appeared to be appropriate for revealing the response capacities at this early age and was shown to be sensitive to changes in the acceptance of flavored substances (Arias, et al., 2007; Miranda-Morales, Nizhnikov, & Spear, 2014). This experiment was conducted at the laboratory of Norman E. Spear at the Center for Development and Behavioral Neuroscience (Binghamton University, SUNY) where the reliability of this technique had been established.

The aim of the experiment was to test whether prenatal exposure to vanilla increases operant responding to this flavor in pups on PD 5 when compared to subjects prenatally exposed to water. Since all these subjects were also evaluated on PD 14, for those subjects tested with vanilla on PD 5, this session served as a "reminder" of the prenatal experience.

Specific Procedures

Subjects were evaluated in an operant chamber at the same ages as in Experiment 1. The first evaluation was on PD 5 and the second evaluation was carried out on PDs 14, 15, 16, and 17 (PDs 14, 15 and 16 were considered as training, and PD 17 as the test day) In both cases we followed the procedures explained in the General Method section.

Experimental design

Ninety-three subjects from 16 litters took part in this experiment. Subjects were quasi-randomly distributed into 12 groups defined by: Prenatal treatment (vanilla or water), the Test substance on PD 5 (vanilla, water, UT) and Conditioning (paired, P, and yoked, Y) (table 3).

Prenatal Treatment	Test on PD5	Test on PD 14 - 17	Condition
Water	Water	Vanilla	P
			y
	Vanilla	Vanilla	P
			y
	UT	Vanilla	P
			y
Vanilla	Water	Vanilla	P
			y
	Vanilla	Vanilla	P
			y
	UT	Vanilla	P
			y

Table 3: Experimental design of Experiment 2. UT: untreated group; P: Paired group; Y: Yoked group.

For the PD 5 test, data were analyzed by a factorial ANOVA (2 x 2 x 2) with the independent variables Prenatal treatment (water or vanilla), Test substance (water or vanilla), and Conditioning (P and Y), while number of sensor touches was the dependent measure. Group UT was not included in the PD 5 test analysis, since no data were collected at this age.

To analyze data from the operant test conducted on PDs 14, 15, 16 and 17, factorial ANOVAs were run separately for each day (training days and test). Each factorial ANOVA (2 x 3 x 2) included Prenatal treatment (water or vanilla), Test substance on PD 5 (water, vanilla or UT), and Conditioning (P and Y) as independent factors, whilst the dependent variable was the number of sensor touches.

Results

A factorial ANOVA conducted on the operant test data on PD 5 yielded significant effects of Prenatal treatment $F(1,77) = 11.82, p < 0.001$, Test substance $F(1,77) = 4.24, p < 0.05$, and Conditioning, $F(1,77) = 11.31, p < 0.001$. Further, the following four significant interactions between variables were found: Prenatal treatment x Test substance $F(1,77) = 9.85, p < 0.005$; Prenatal Treatment x Conditioning $F(1,77) = 4.32, p < 0.05$; Test substance x Conditioning $F(1,77) = 7.48, p < 0.01$, and Prenatal treatment x Test substance x Conditioning $F(1,77) = 9.97, p < 0.01$. The post hoc test of the three-way interaction

revealed that subjects prenatally treated with vanilla and tested with vanilla, from the paired condition, responded more to vanilla than the remainder of the subjects (Figure 3).

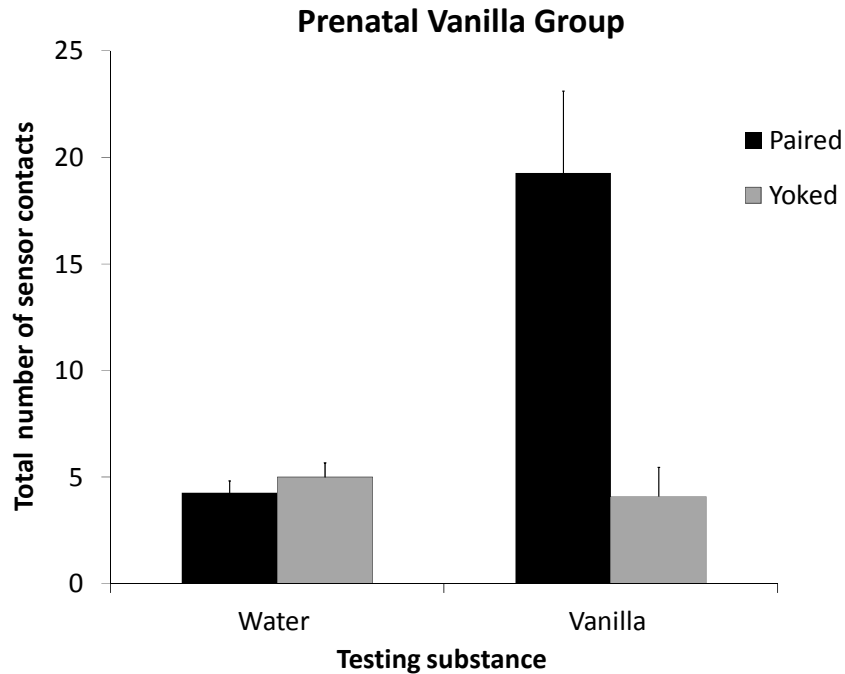


Figure 3. Total number of sensor contacts realized by the subjects prenatally exposed to vanilla. Half of the subjects responded to water and the other half to vanilla.

The ANOVAs conducted on data collected on PDs 14-17 yielded no significant effect of any variable, showing that the response of all subjects was the same, independent of the prenatal exposure, the conditioning, and the solution received on PD 5 (see figure 4).

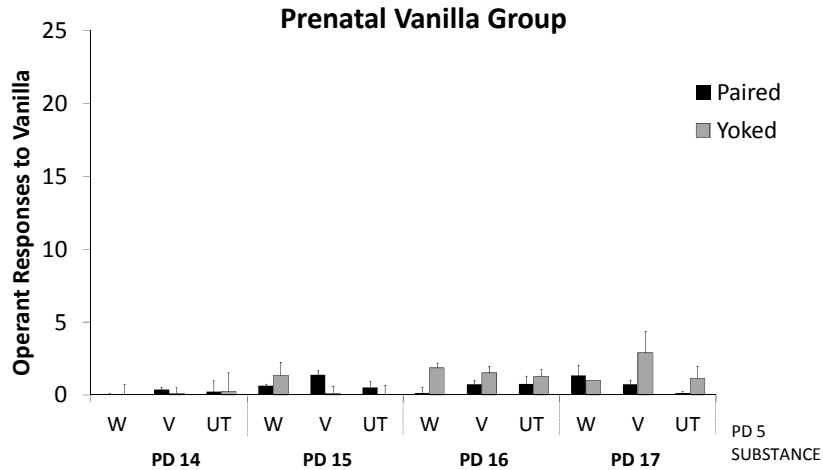


Figure 4. Total number of sensor contacts realized by the subjects prenatally exposed to vanilla. Half of the subjects responded to water and the other half to vanilla.

In contrast with the results obtained in the previous experiment, the present findings confirm that after prenatal exposure to vanilla, pups on PD 5 respond more to this substance compared with pups that had received no prenatal experience with this substance. Therefore, we can also confirm that the intake evaluation itself is not sensitive enough at this age because it does not permit the observation of differential responses to the substances experienced before birth. Nonetheless, the theoretical implications of the positive results observed in this experiment are substantial, since they demonstrate that prenatal exposure to a flavor, without any explicit reinforcement, is enough to change the postnatal response to this substance. However, the null results obtained

when testing on PD 14-17 suggests that the prenatal memory of the experienced substance does not persist until this age, even in the group that was re-exposed on PD 5.

EXPERIMENT 3

In 2014, Mendez-Gallardo and Robinson published an important study describing a new technique named the *odor-induced crawling locomotion test* for evaluating newborn rats. It became clear that this technique, which was completely non-invasive and required minimal maternal separation, was the optimal procedure to evaluate the effects of prenatal exposure to flavors immediately after birth. We therefore adopted this new technique to test the attractiveness of the odors of vanilla and alcohol in 1-day old pups exposed prenatally to these substances.

Specific procedures and experimental design

On GD 17-20 nineteen pregnant dams were treated, six were administered with vanilla, another seven received alcohol, and the remaining six dams were administered with water, as described in the General Methods section. Six neonates from each of these litters were tested on PD 1 - a female and a male - with one of the testing odors: vanilla, alcohol and water. A total of 114 neonates were evaluated, although data from 113 subjects were analyzed, since scores from one pup treated prenatally with vanilla and tested with alcohol were excluded due to an experimental error. In sum, the experimental design resulted in 9 groups assigned

according to Prenatal treatment (vanilla, ethanol or water) and Test odor (vanilla, ethanol or water).

Results

Data from this experiment have already been published in the *Journal of Developmental Psychobiology* (Gaztañaga, Aranda, & Chotro, 2015). The results are depicted in Figure 5. A factorial ANOVA conducted on the data of distance travelled indicated a significant effect of Test odor $F(2,95) = 8.78$ $p < 0.001$, and a significant interaction between Test odor and Prenatal treatment, $F(4,95) = 8.20$ $p < 0.001$. The post hoc analyses revealed that pups exposed prenatally to water did not show significant differences in response to any of the tested odors ($p > 1$). However, pups exposed prenatally to vanilla crawled a significantly further distance towards the vanilla odor than to either ethanol or water. Similarly, pups exposed in utero to ethanol crawled a longer distance towards the ethanol odor than to vanilla or water. It was also observed that pups prenatally exposed to alcohol travelled a longer distance towards the odor of alcohol when compared to pups tested with this same substance but exposed prenatally to vanilla or water. In the same way, pups from vanilla-treated dams crawled longer towards the vanilla odor than pups from the remaining two odor treatments (all p 's < 0.001).

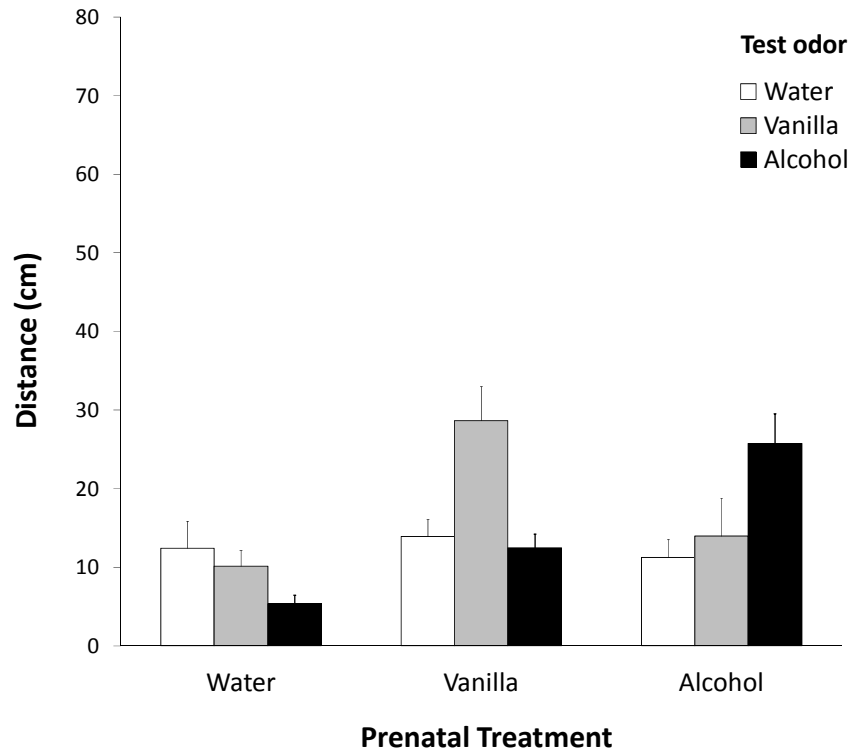


Figure 5. Distance (cm) travelled by the newborn rats after water, vanilla or ethanol odor and as a function of prenatal treatment (water, vanilla, or ethanol).

The results of the present experiment confirm that, at least when tested in the neonatal period and with a technique appropriate for their age, rats given prenatal exposure to a non-ethanol flavor during the last days of gestation show an enhanced tendency to accept this substance. With prenatal exposure to ethanol an increased attraction to its odor was also observed, a result in accordance with previous studies testing older animals with different procedures.

EXPERIMENT 4

Using the same testing procedure employed in the previous experiment, the purpose of this experiment was to explore the role of the activity of the opioid receptors in the effect of increased attraction to an odor experienced prenatally and with no pharmacological effects (vanilla). Based on the "KIF theory" our hypothesis was that antagonizing the kappa opioid receptor would reduce the attraction to the prenatally exposed odor, while blocking mu would not have any effect.

Specific procedures and experimental design

In this experiment, we evaluated a total of 96 subjects derived from 16 litters. For the prenatal treatment, on GDs 17-20 four dams received water, four vanilla, another group of four vanilla and NXZ, and another group of four dams was administered with vanilla and NBI, following the procedures described previously. The offspring of these dams were tested on PD 1. On this day six neonates - a female and a male - from each litter were tested with one of the following testing substances: vanilla, alcohol, and water. In sum, subjects were assigned to 12 groups according to Prenatal treatment (water, vanilla, vanilla-NXZ and vanilla-NBI) and Test odor (water, ethanol or vanilla).

Results

Data from this experiment have already been published in the Journal *Developmental Psychobiology* (Gaztañaga, Aranda, & Chotro, 2015). The results of this experiment are shown in Figure 6. A factorial ANOVA conducted on these data revealed significant effects of the variable Test odor $F(2,72) = 15.39$ $p < 0.001$, and an interaction between Test odor and Prenatal treatment $F(6,72) = 2.23$ $p < 0.05$. The post hoc analyses revealed that pups travelled more when tested with vanilla than with alcohol or water, regardless of the Prenatal treatment ($p < 0.001$). The analyses of the interaction revealed that newborns treated with water did not show any difference in distance travelled when tested with any of the three substances. In the group given prenatal exposure to vanilla, pups crawled a further distance towards the vanilla odor than towards ethanol or water (both p 's < 0.001). Pups exposed prenatally to vanilla-NXZ behaved similarly to water-treated controls, i.e. no significant differences were detected between them in terms of distance crawled towards any of the three test odors. In contrast, pups prenatally treated with vanilla-NBI crawled significantly further when tested with vanilla than when tested with alcohol ($p = 0.002$) or water ($p = 0.001$). When comparing between odor treatments, the post-hoc tests revealed that on the test with vanilla, pups treated prenatally with vanilla and vanilla-NBI did not differ, but both crawled significantly longer distances towards vanilla than pups exposed prenatally to water or vanilla-NXZ (vanilla vs. water or

vs. vanilla-NXZ, p 's < 0.001; vanilla-NBI vs. water, p = 0.005 and vanilla-NBI vs. vanilla-NXZ, p = 0.009). No significant differences were detected between water and vanilla-NXZ treated groups.

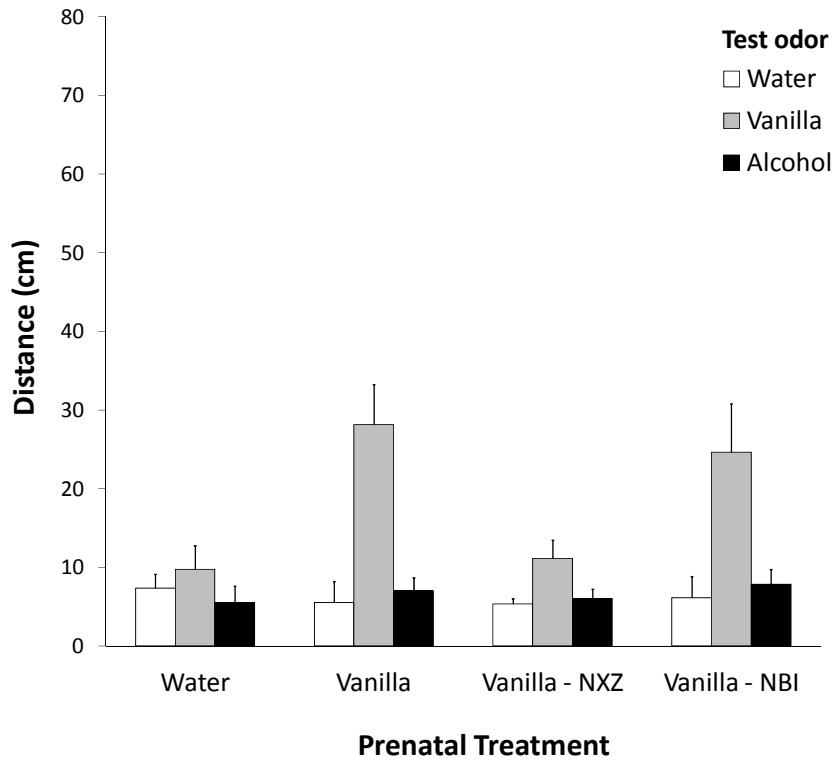


Figure 6. Distance (cm) travelled by the newborn rats after water, vanilla or ethanol odor and as a function of prenatal treatment (water, vanilla, vanilla together with Naloxonazine and vanilla together with Nor-binaltorphimine).

In the current experiment we have replicated our previous finding of an increased attractiveness to vanilla. The

new findings of interest are that blocking the mu opioid receptor system annulled the enhanced attraction effect to the odor of vanilla after its prenatal exposure, whilst treatment with the kappa opioid receptor antagonist had no effect on this response, since pups with this odor treatment continued displaying the enhanced response to the vanilla odor. The outcomes with the antagonists were, surprisingly, quite the opposite of those anticipated by our hypothesis. The different explanations of these findings (along with the remainder of the results of this thesis) will be developed in the General Discussion section.

EXPERIMENT 5

In this experiment, we tested the neonatal response of pregnant dams to the odors of vanilla, alcohol, or water, after administering mu and kappa opioid receptor antagonists along with alcohol. When compared with controls, we expected to see a decrease in the attractiveness of the ethanol odor in pups exposed to the drug together with the antagonists. And on the basis of previous experiments conducted in this laboratory, we expected this effect to be particularly marked in those receiving the mu opioid receptor antagonist (Diaz-Cenzano, et al., 2014).

Specific procedures and experimental design

In this experiment, a total of 96 subjects derived from 16 litters were evaluated. For the prenatal treatment, on GDs 17-20, four dams received water, four received ethanol, another group of four received ethanol and NXZ, and a further group of four was administered with ethanol and NBI, following the procedures described previously. The offspring of these dams were tested on PD 1. On the test day, six neonates, a female and a male, from each litter were tested with one of the following test substances: vanilla, alcohol, and water. In sum, subjects were distributed among 12 groups according to Prenatal treatment (water, ethanol, ethanol-NXZ and ethanol-NBI) and Test odor (water, ethanol, or vanilla).

Results

A factorial ANOVA conducted on the data from the distance crawled yielded significant effects of the variables Test odor $F(2,72) = 12.73$ $p < 0.001$; and Prenatal treatment, $F(3,72) = 5.79$ $p < 0.001$, in addition to a significant interaction between these two variables $F(6,72) = 4.22$ $p < 0.001$. Post-hoc analyses exploring this interaction revealed that pups exposed prenatally to ethanol crawled a further distance towards the ethanol odor than to either vanilla or water ($p < 0.001$), as well as in comparison to neonates prenatally treated with either water, ethanol-NXZ or ethanol-NBI ($p < 0.001$). In addition, pups with Prenatal treatment with ethanol-NBI crawled a longer distance towards the alcohol odor than pups with prenatal exposure to water ($p = 0.030$). All other possible comparisons were not significant.

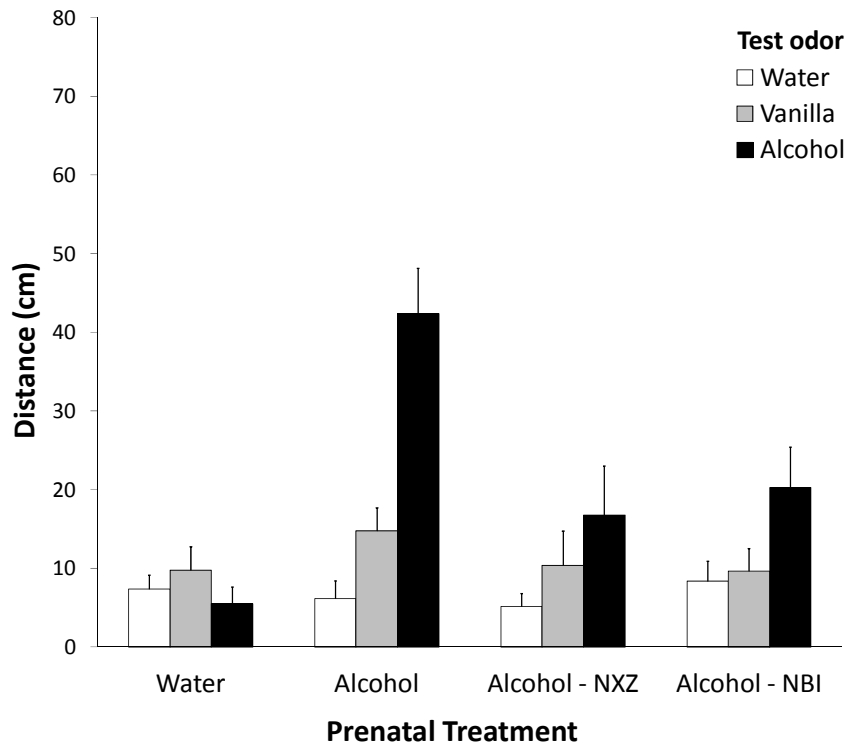


Figure 7. Distance (cm) travelled by the newborn rats towards water, vanilla, or ethanol odor as a function of prenatal treatment (water, ethanol, ethanol-NXZ and ethanol- NBI).

These data replicate the results obtained in Experiment 5, i.e. prenatal exposure to ethanol enhances the attractiveness of the preexposed odor, an effect that can be detected using the crawling technique soon after birth. Administering this substance together with the mu opioid receptor antagonist completely blocked this effect, since treated pups exhibited similar behavior to those that had never received exposure to ethanol (water control). In the

group exposed to ethanol and kappa opioid receptors, a significant reduction in the attractiveness of ethanol odor was observed, when compared with pups exposed to ethanol. However, since this group still displayed some attraction to the ethanol odor relative to pups treated with water, it can be deduced that the kappa opioid system also plays as a role in the prenatal ethanol exposure effect.

EXPERIMENT 6

This is a first experiment of a series designed to address the role of ethanol's first metabolite -acetaldehyde- in the reinforcing effects of prenatal ethanol exposure. In this experiment this was assessed using a sequestering agent of acetaldehyde (D-penicillamine, DP), which was administered prenatally together with ethanol. The pups were then evaluated on PD 5 in an operant conditioning task. As in Experiment 2, this was conducted at the laboratory of Norman E. Spear at the Center for Development and Behavioral Neuroscience (Binghamton University, SUNY) where the successful use of this technique had previously been established.

Specific procedures and experimental design

In the current experiment we tested 160 subjects derived from 20 dams. The experimental design resulted in 8 groups that were assigned according to Prenatal ethanol (ethanol or water), Prenatal DP (DP or saline), Test substance (saccharin or ethanol), and Conditioning (P or Y). The resulting groups were referred to as: ethanol-DP-P, ethanol-DP-Y, ethanol-saline-P, ethanol-saline-Y, water-DP-P, water-DP-Y, water-saline-P, and water-saline-Y. The duration of operant training was 15 minutes, but unlike the previous experiments, in this case we added a 6-minute extinction session. In the

extinction phase the infusion pump was disconnected so that the pups did not receive the substance every time pups from condition P touched the sensor. This phase was added to confirm that the P subject had acquired the operant conditioning response.

Results

Data obtained from both tests (saccharin and ethanol) were analyzed separately with factorial ANOVA ($2 \times 2 \times 2$) for training and for extinction. Results from the training and extinction tests with saccharin showed neither significant main effects nor interactions between the analyzed variables (data not shown). This means that, during both training and extinction, subjects from all of the prenatal treatments responded similarly when saccharin was the reinforcer, independent of conditioning.

However, a factorial ANOVA conducted on the training date with ethanol (Figure 8) revealed significant effects of Prenatal DP, $F(1,72) = 10.73$, $p < 0.001$, and of Conditioning $F(1,72) = 26.50$, $p < 0.001$; as well as an interaction between both variables $F(1,72) = 13.13$, $p < 0.001$, an interaction between Prenatal ethanol and Conditioning $F(1,72) = 7.49$, $p < 0.001$, and a triple interaction between Prenatal ethanol x Prenatal DP x Conditioning $F(1,72) = 5.69$, $p < 0.01$.

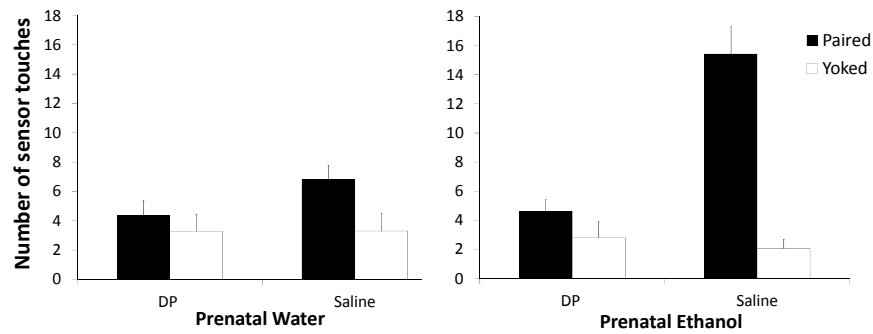


Figure 8: On the left, total number of responses (sensor contacts) during training with ethanol as the Test substance emitted by subjects treated prenatally with water, as a function of Prenatal DP (saline or DP), and Conditioning (Paired or Yoked). On the right, total number of responses (sensor contacts) during training with ethanol as the Test substance emitted by subjects prenatally treated with ethanol as a function of Prenatal DP (saline or DP), and Conditioning (Paired or Yoked).

In the extinction phase the ANOVA indicated again a significant effect of Prenatal ethanol $F(1,72) = 8.26, p < 0.005$, Prenatal DP $F(1,72) = 5.45, p < 0.05$, Conditioning $F(1,72) = 8.95, p < 0.005$, as well as interactions between Prenatal Ethanol and Conditioning $F(1,72) = 4.42, p < 0.05$, Prenatal DP and Conditioning $F(1,72) = 15.04, p < 0.001$, and the triple interaction between these three variables $F(1,72) = 3.36, p < 0.05$ (Figure 9).

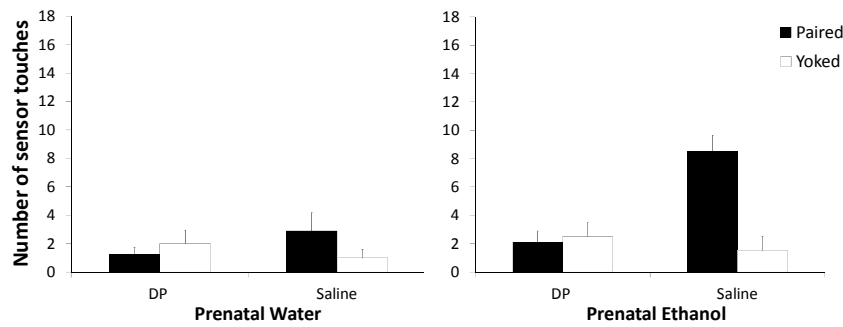


Figure 9: On the left, total number of responses (sensor contacts) during extinction with ethanol as the Test substance emitted by subjects treated prenatally with water, as a function of Prenatal DP (saline or DP), and Conditioning (Paired or Yoked). On the right, total number of responses (sensor contacts) during extinction with ethanol as the Test substance emitted by the subjects prenatally treated with ethanol as a function of Prenatal DP (saline or DP), and Conditioning (Paired or Yoked).

Subsequent analyses of the triple interaction obtained when analyzing the ethanol training data revealed that ethanol-saline-P subjects responded significantly more than their ethanol-saline-Y controls ($p < 0.001$), and also significantly more than the remaining P subjects (water-saline-P, water-DP-P and ethanol-DP-P). When analyzing the triple interaction obtained during the extinction phase, a similar profile of results was obtained. These results indicate that sequestering acetaldehyde during prenatal ethanol exposure abolishes the effect of the enhanced acceptance for ethanol after birth.

EXPERIMENT 7

In this experiment our aim was to replicate the previous experiment but evaluating pups on PD 14 with two tests: an intake test and a taste reactivity test (TRT). On the basis of the findings obtained in operant learning, in this experiment we anticipated a decrease in ethanol intake and palatability in the group prenatally administered with ethanol together with D-penicillamine.

Specific procedure/Experimental design

For this experiment, we tested 80 subjects derived from 20 dams were, 40 on an intake test and 40 on a TRT. In both cases, the experimental design resulted in 4 groups arranged according to Prenatal ethanol condition (ethanol or water), and the Prenatal DP (DP or saline). The resulting groups were referred to as: ethanol-DP, ethanol-saline, water-DP, and water-saline. Subjects evaluated by intake were first given a water test and an hour later an ethanol test. Pups evaluated on TRT received two minutes of water (W1-W2) followed by five minutes of ethanol (E1-E5).

Results

A factorial ANOVA (2x2) conducted on the data from the water intake test on PD 14 revealed no significant differences. However, when we analyzed the ethanol intake data a significant effect of Prenatal DP $F(1,36) = 10.67, p < 0.002$ was found, along with an interaction between Prenatal ethanol and Prenatal DP $F(1,36) = 6.76, p < 0.05$. The post hoc analyses revealed that subjects given the prenatal ethanol treatment consumed more than the rest of the groups, including the subjects in Group ethanol-DP, who consumed ethanol at the same level as the control groups water-DP and water-saline (Figure 10).

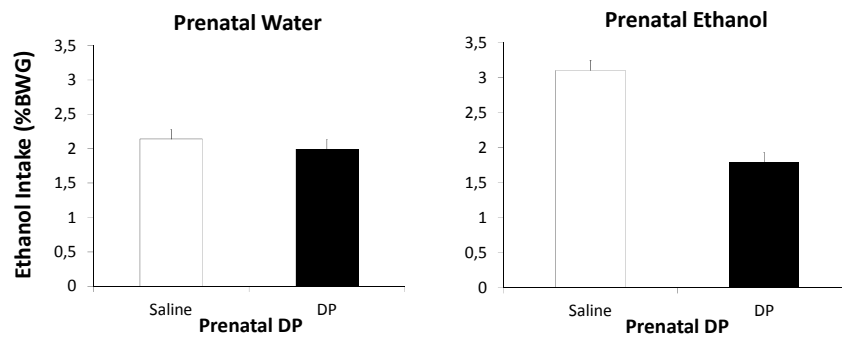


Figure 10. On the left mean intake (%BWG) of ethanol of subjects prenatally treated with water as a function of prenatal DP (saline or DP). On the right, mean intake (%BWG) of ethanol of subjects prenatally treated with water as a function of prenatal DP (saline or DP).

Further, the data obtained on the TRT was analyzed using two repeated-measure ANOVAs - one for the appetitive reactions and the other for the aversive reactions. Unexpectedly, there were no significant main effects or interactions for the appetitive responses to ethanol (Figures 11 and 12). Even though subjects exposed prenatally to ethanol showed an increased consumption of ethanol, they did not show significantly more appetitive responses to this substance, and therefore, there was no scope to see a reduction in palatability following DP prenatal treatment.

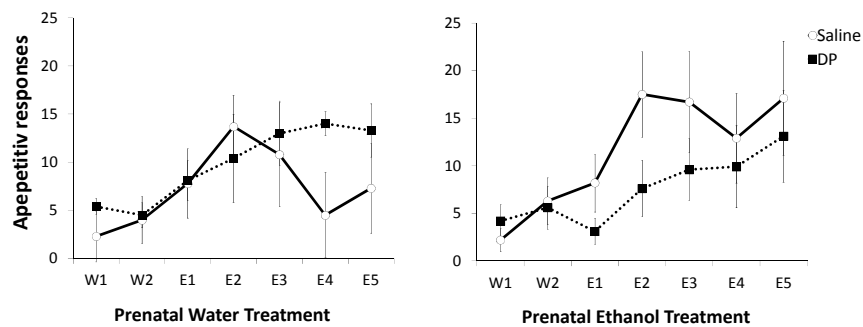


Figure 11. On the left, mean duration (sec) of appetitive responses to water (W1 and W2) (and ethanol (E1-E5) Of subjects prenatally treated with water as a function of Prenatal DP (saline or DP) treatment. On the right, mean duration (sec) of appetitive responses to ethanol (W1 and W2) (and ethanol (E1-E5) Of subjects prenatally treated with water as a function of Prenatal DP (saline or DP) treatment.

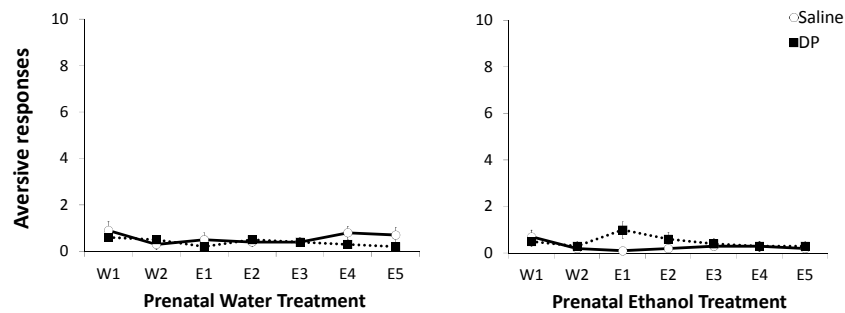


Figure 12. On the left, mean duration (sec) of aversive responses to water (W1 and W2) (and ethanol (E1-E5) Of subjects prenatally treated with water as a function of Prenatal DP (saline or DP) treatment. On the right, mean duration (sec) of aversive responses to ethanol (W1 and W2) (and ethanol (E1-E5) Of subjects prenatally treated with water as a function of Prenatal DP (saline or DP) treatment.

EXPERIMENT 8

In this final experiment we continued to explore the role of acetaldehyde in the prenatal ethanol exposure effect. Given that on PD 14 we found results that were partially conclusive, i.e. a decrease in ethanol intake when administering DP, whilst failing to find an effect on TRT, we decided to evaluate this with the odor-induced crawling locomotion test on PD1. This evaluation technique has shown to be sensitive enough for detecting prenatal memories in our previous experiments. In addition, we also evaluated the effect of D-penicillamine when administered together with a substance with flavor but no pharmacological effect i.e., vanilla.

Specific procedures and experimental design

In this experiment we tested 144 subjects derived from 24 dams. The design resulted in 18 groups arranged according to Prenatal odor (ethanol, vanilla, or water), the Prenatal DP (DP or saline) and Test odor (ethanol, vanilla, or water). Maintaining this same sequence of variables, the groups were referred to as: ethanol-DP-ethanol, ethanol-DP-vanilla, ethanol-DP-water, ethanol-saline-ethanol, ethanol-saline-vanilla, ethanol-saline-water, vanilla-DP-ethanol, vanilla-DP-vanilla, vanilla-DP-water, vanilla-saline-ethanol, vanilla-saline-vanilla, vanilla-saline-water, water-DP-ethanol, water-DP-

vanilla, water-DP-water, water-saline-ethanol, water-saline-vanilla, and water-saline-water.

Results

A factorial ANOVA ($3 \times 2 \times 3$) conducted on the test data revealed significant effects of Prenatal odor $F(2,126) = 14.55$, $p < 0.001$, and Test odor $F(2,126) = 28.73$, $p < 0.001$. An interaction between these two variables was also obtained $F(4,126) = 24.03$, $p < 0.001$, and also between variables Prenatal odor and Prenatal DP $F(2,126) = 6.43$, $p < 0.005$. Finally, a triple interaction was found between Prenatal odor, Prenatal DP, and Test odor $F(4,126) = 5.90$, $p < 0.001$. The post-hoc analyses showed that group ethanol-saline-ethanol crawled a longer distance towards the ethanol odor than any other group. This difference, however, was abolished by the administration of D-penicillamine together with ethanol, since the distance crawled by Group ethanol-DP-ethanol did not differ from the remaining controls. However, pups from group vanilla-saline-vanilla and vanilla-DP-vanilla displayed a similar attraction to the vanilla odor in comparison with the other odors. In addition, these two groups differed significantly from the groups exposed prenatally to ethanol or water (Figure 13, 14 and 15).

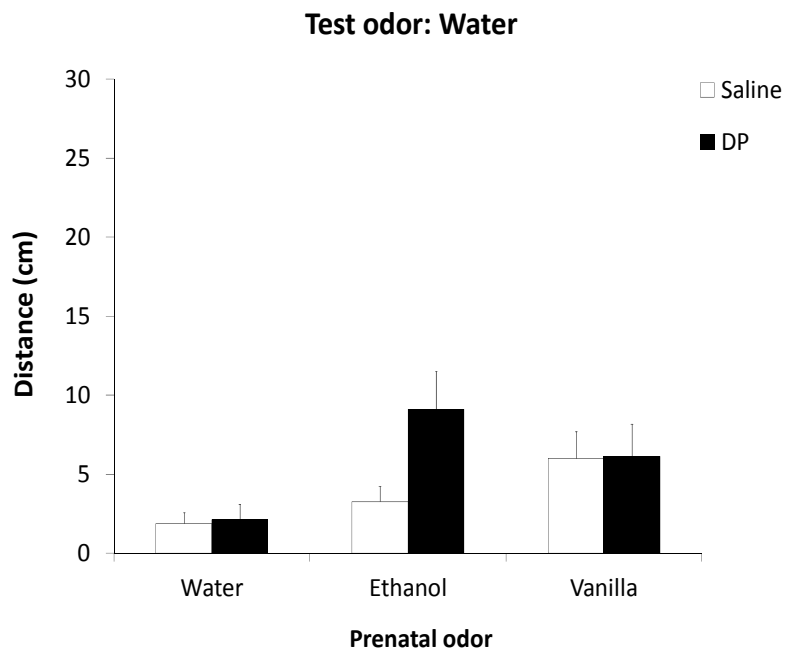


Figure 13. The distance crawled (cm) towards the odors of water as a function of Prenatal odor (water, ethanol or vanilla) and Prenatal DP (saline or DP).

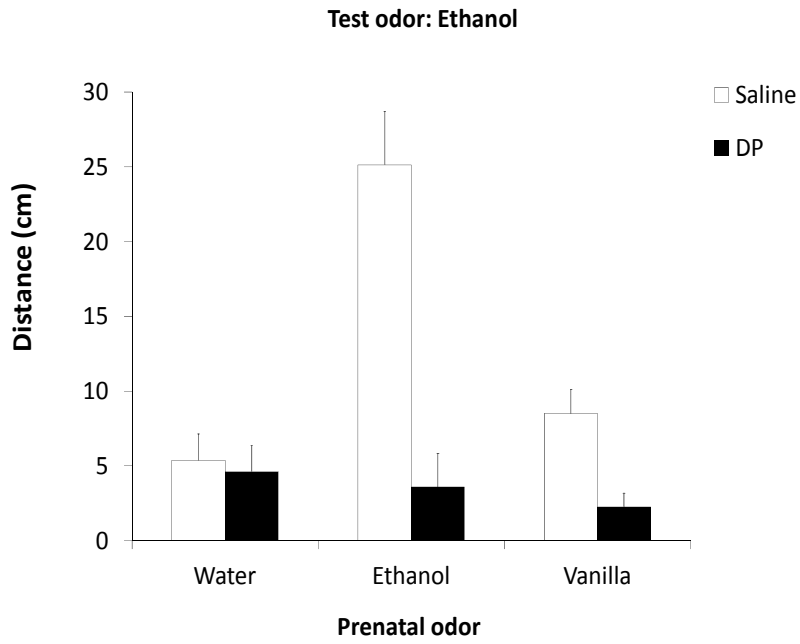


Figure 14. The distance crawled (cm) towards the odors of ethanol as a function of Prenatal odor (water, ethanol or vanilla) and Prenatal DP (saline or DP).

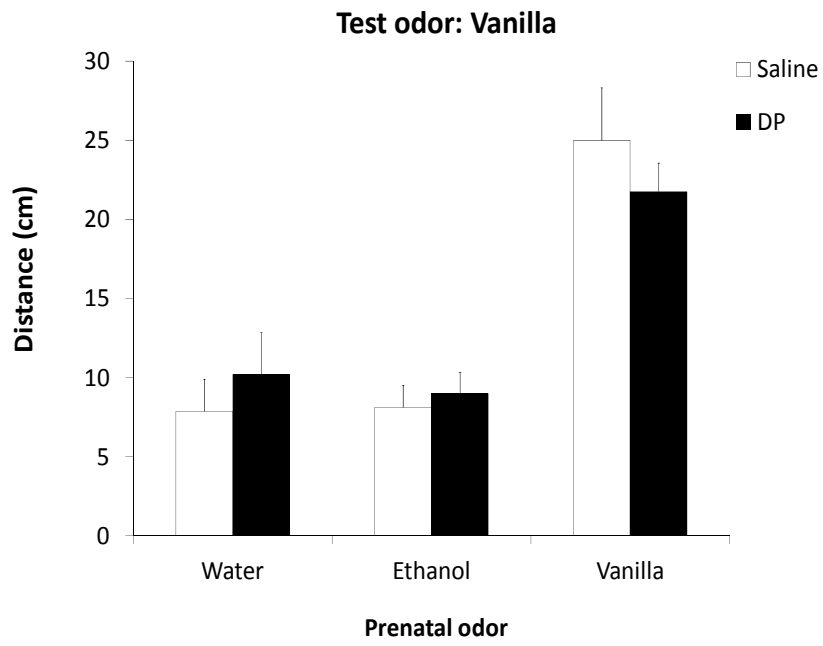


Figure 15. The distance crawled (cm) towards the odors of vanilla as a function of Prenatal odor (water, ethanol or vanilla) and Prenatal DP (saline or DP).

DISCUSSION



The general objective of this work was to conduct an in-depth investigation into the possible role of the opioid system and the amniotic fluid in the prenatal ethanol exposure effect, along with examining the importance of the first metabolite of this drug in generating this effect. The first three experiments were designed with the purpose of testing if prenatal exposure to substances with no pharmacological effects would have a similar effect to that of exposure to ethanol, i.e. increased acceptance. The other two experiments were conducted to delve deeper into the role of the kappa and mu opioid receptor systems in the increased acceptance of substances exposed prenatally. Finally, the last three experiments were focused on the role of acetaldehyde on the effects observed after prenatal ethanol exposure.

Taken together, the results of all the experiments show that infants exposed prenatally to vanilla, when tested 2 weeks after birth, did neither increase their intake of this substance nor augmented their operant response when vanilla was the reinforcer, even when subjects had received a reminder on PD 5 (Experiment 1). When tested at an earlier stage of development - on PD 5 - no effects were detected in terms of intake (Experiment 1). However, an increased operant response to vanilla flavor was observed at this age (Experiment 2). Further, when tested on PD 1, neonates preexposed to vanilla displayed a greater attraction to vanilla odor (Experiment 3). This enhanced attraction effect with vanilla was related to the fetus' opioid system, since the effect was

abolished by blocking the mu - but not the kappa - opioid receptor system during prenatal exposure to vanilla (Experiment 4). However, the attraction to the ethanol odor observed in neonates exposed prenatally to this drug was completely abolished by antagonizing mu opioid receptors, and partially reduced by blocking kappa receptors (Experiment 5). In the final experiments it was observed that acetaldehyde plays an important role in the effects of enhanced attraction for ethanol after its prenatal exposure. Its elimination impeded the observation of an attraction to the odor of ethanol on PD 1 (Experiment 6), of the enhanced operant responses reinforced with ethanol on PD 5 (Experiment 7), and of the increased ethanol consumption on PD 14 (Experiment 8).

The lack of a positive result on the intake test in Experiment 1 agrees in part with the results obtained by Díaz-Cenzano et al., (2014), in which prenatal exposure to non-pharmacological flavored substances, such as anise or vanilla, did not induce any differential response in terms of intake or palatability to those same flavors when tested on PD 14. However, as the authors suggested, the lack of an increased acceptance on PD 14 does not rule out the possibility that the amniotic fluid (with its component KIF) plays a role in prenatal flavor learning, and it might be possible to detect the effect if the subjects are tested closer to birth. Accordingly, in Experiment 1 pups were also evaluated on PD 5, but no effects were detected with this measure. Moreover, when using this

early evaluation as a “reminder” no effects were detected on PD 14. These results run counter to both our hypothesis and some of the findings of classic studies showing a preference for the food experienced in utero, for example for carrot-flavored food in babies prenatally exposed to carrot (Menella et al., 2001), and for garlic after fetal experience with that flavor (Hepper, 1998). Furthermore, the results of this experiment seem to disagree with the hypothesis stated by Robinson and collaborators (2010), in which a component of the amniotic fluid of GD 20, KIF, is responsible for the heightened postnatal response to the prenatally experienced flavors. However, before completely rejecting the idea that fetuses acquire a conditioned appetitive response to a flavor reinforced by KIF, we have to consider the possibility that this conditioned response was not strong enough to be retained until PD 14, even after re-exposure to the flavor on PD 5. This could also supply an explanation for the lack of a differential response on PD 5, given that there was a 6-day interval between the final exposure to vanilla (GD 20) and testing on PD 5, an interval that could be too long for learning to persist, at least for subjects at this developmental stage. A further possibility is that the intake test procedure is not sensitive enough to allow the detection of this learned response to vanilla at this early age. Although this technique has been successfully used repeatedly in our (and other) laboratories to test intake at different preweaning stages, it had never been employed to test subjects before PD 8. Therefore, we could

not be entirely certain of whether or not pups were able to effectively reject and swallow the substance administered intra-orally. These questions were addressed more satisfactorily in the following two experiments.

The results of Experiments 2 and 3 - obtained using two different techniques more appropriate for evaluating pups closer to birth – allowed us to confirm the finding of a differential response to vanilla after prenatal exposure to this flavor. The increased operant responses on PD 5 and attractiveness to vanilla on PD 1 observed after its prenatal exposure on GD 17-20 constitute our first evidence of appetitive learning acquired in utero with a non-ethanol substance. These data appear to support our initial hypothesis posed for these experiments and confirm the importance of evaluating subjects closer to birth to detect learning occurring prenatally. It should be noted that the decision to evaluate subjects using different techniques was also critical for detecting the expression of prenatal learning, demonstrating that operant responses (on PD 5) and crawling (on PD 1) are more sensitive techniques than the intake test for detecting these effects. The results also appear to support the hypothesis proposed by Robinson and collaborators., which suggests that the amniotic fluid present in the final days of gestation contains an agent (KIF) that activates the opioid system of the fetus, acting as an appetitive reinforcer and allowing appetitive conditioning of flavors experienced prenatally. As they stated, any substance - not only ethanol -

exposed on those days of gestation would be more accepted by the pups. The finding on PD 1 and 5 also agrees with the reports of a preference or heightened intake of flavors experienced prenatally (Hepper, 1998; Menella et al., 2001; Schaal et al., 2000). However, when using the operant technique to measure the responses on PD 14 no differential responses were obtained. This latter finding suggests that the memory of the prenatal experience is retained and expressed in the neonatal period but does not persist until the rat's infancy, as in the case of prenatal exposure to ethanol. In this case, the experience with ethanol in utero affects the response not only in infancy (Díaz-Cenzano et al., 2012; Miranda-Morales, 2012), but also in adolescence and adulthood (Chotro, et al., 2007). A possible explanation for the long lasting effect of the ethanol prenatal experience in comparison with the effects found with vanilla, could relate to the effect of ethanol on the activation of the opioid receptor system. This system could play a critical role not only in the acquisition of an appetitive memory but also in its retention. In this regard, previous experiments conducted in this laboratory have shown that the administration of a general antagonist of opioid receptors (naloxone) or a specific antagonist for mu-opioid receptors (naloxonazine) completely abolished the enhanced intake and palatability usually observed on PD 14, whilst administering a specific kappa opioid receptor antagonist, reduced the palatability of ethanol but not the enhanced intake response (Díaz-Cenzano et al., 2014). These results

suggest that it is the mu-opioid receptor system that is chiefly responsible for the increased acceptance response to ethanol acquired in utero.

Contrary to our expectations, the results of Experiment 4, demonstrated that the KIF component of the amniotic fluid plays no significant role in the increased attractiveness to the odor of vanilla after its prenatal exposure. In particular, antagonizing the kappa opioid receptors failed to modify the pup's response to vanilla, whereas antagonizing mu opioid receptors was successful in altering this response. Given that these results do not provide support for the "KIF hypothesis", we need to find alternative explanations for the effect of increased attraction to vanilla after its prenatal exposure. One possibility could be a non-associative effect of familiarity or a mere stimulus exposure effect, which would induce an enhanced attraction on PD 1 and PD 5 for the odor experienced in utero, and which fails to persist over time. In contrast, an alternative approach may be needed to explain the role played by the mu-opioid receptor system. One possible explanation is related to the acute behavioral effects of antagonizing mu-opioid receptors, which reduce consummatory behaviors, such as mouthing, licking, and the intake of substances. This has been described in adult rats (Berridge, 2000), in preweaning rats (Arias et al., 2010) as well as in fetuses (Smotherman & Robinson, 1995). It is conceivable that while under the effects of the mu opioid antagonist, there is a reduction or an inhibition of the mouth movements of the

fetus, and thus a reduction of ingestion of the amniotic fluid, diminishing the likelihood of sampling and perceiving its chemosensory properties and hence reducing the fetal experience with the stimuli. In other words, blocking mu opioid receptors may impede the fetal perception of the odor of vanilla, and, possibly, of any flavor present in the fetal environment.

In the case of ethanol prenatal exposure, the outcome of antagonizing the kappa and mu opioid receptors (Experiment 5) suggests that the mu opioid subsystem could be critical in prenatal learning, whereas the kappa opioid subsystem appears to be only partially implicated in this process. Blocking mu opioid receptors completely abolished the effect of enhanced attractiveness to the odor of ethanol, since in this case pups behaved as if they had never received prior exposure to the substance. Antagonizing the kappa opioid receptors, however, significantly reduced the attractiveness of the ethanol odor, although this effect was not as dramatic as that observed when blocking mu receptors. It can be assumed, therefore, that the kappa opioid system also plays a role in generating the prenatal ethanol exposure effect. This finding is compatible with those obtained by Díaz-Cenzano et al., (2014) when testing preexposed pups two weeks after birth on both intake and taste reactivity tests.

Taken together, the results obtained after vanilla and ethanol prenatal exposure with kappa or mu opioid receptor

antagonists suggest that the mu subsystem plays a crucial role in appetitive prenatal learning; in particular, antagonizing this system blocks all expression of learning by the neonates on PD 1 with both flavors. In the case of ethanol, this outcome was expected, given that the reinforcing effects of ethanol in adult rats are mainly mediated by the mu-opioid receptor system (Stromber, Casale, Volpicelli, Volpicelli, & O'Brien, 1998); and that similar results have been obtained in previous studies (Arias & Chotro, 2005; 2006; Chotro & Arias, 2003; Díaz-Cenzano et al., 2010; 2014; Miranda-Morales et al., 2012; 2014). In the case of vanilla, however, the results were surprising, and there are at least two alternative explanations of this unexpected outcome. Whilst there is no direct evidence in support of this suggestion, one possibility is that vanilla activates the mu opioid system. Alternatively, blocking mu receptors affects prenatal learning in some way. As mentioned previously, the acute effects of blocking mu receptors could interfere with the perception of the conditioned stimulus by inhibiting consummatory behaviors. In addition, the opioid system has been found to play a role in the acquisition of associative learning. Further, the results of some studies have demonstrated that opioid inhibition both before and after training impairs acquisition of learning in procedures such as habituation learning, shuttle-avoidance, and cued fear conditioning in adult rats (Izquierdo, 1980; McNally, Pigg, & Weidemann, 2004; Meilandt, Barea-Rodriguez, Harvey, & Martinez, 2004; Messing, Allen, Aanonsen,

& Sparber, 1989), as well as extinction in developing rats (Kim and Richardson, 2009). On the basis of these findings, it might be possible to argue, that blocking the opioid system prevents the acquisition of learning by the rat fetus. However, this explanation is not entirely satisfactory since some studies have reported the opposite finding.. For instance, mu-opioid activation has been found to retard the acquisition of delayed eye-blink conditioning in rabbits (Aloyo, Romano, & Harvey, 1993). In conclusion, the mu-opioid receptor system appears to play a critical role in observing both enhanced acceptance of ethanol after prenatal exposure, and the increased attractiveness for a vanilla odor observed in neonates prenatally exposed to this flavor. In contrast, the kappa opioid receptor system appears to be involved only in the effects of prenatal ethanol exposure.

The final three experiments of this thesis were concerned with the role played by acetaldehyde in appetitive learning acquired in utero. The results confirmed our expectations. In particular, we have found that in the absence of acetaldehyde, prenatal ethanol exposure fails to produce an increased acceptance of ethanol on PD 14. In addition, this substance is not able to serve as a reinforcer in an operant conditioning paradigm on PD 5, and does not become particularly attractive for PD 1 neonates. These results suggest that the prenatal ethanol exposure effect of enhanced acceptance for ethanol is completely blocked when acetaldehyde is sequestered, thus suggesting that

acetaldehyde is vital for the reinforcing effects of ethanol. This conclusion is also in agreement with studies by Karahanian et al., (2011) and Quertemont & Tambour, (2004) in which they highlight the essential role of ethanol's first metabolite in the reinforcing effects of the drug. Further, the results are in accordance with other studies conducted in adult rats (using a variety of behavioral measures) in which D-penicillamine was used to eliminate acetaldehyde. For instance, voluntary drinking of ethanol is decreased by the administration of this drug (Font et al., 2006), operant ethanol self-administration is reduced (Peana et al., 2015), ethanol relapse-like drinking is prevented (Martí-Prats et al., 2015), and anxiolytic effects produced by moderate doses of ethanol are abolished (Correa et al., 2008). If we focus on studies with infant or neonatal rats, very few have analyzed the role of acetaldehyde. The most recent studies have shown that acetaldehyde may act as an unconditioned stimulus in a similar way to ethanol, and also that D-penicillamine abolishes conditioned responses learned with both ethanol and acetaldehyde as the US (March et al., 2013; Pautassi et al., 2011). The results of the final experiments of this thesis also confirm the relevance of centrally produced acetaldehyde, as opposed to peripheral acetaldehyde. As mentioned in the introductory section, within the fetal context, due to the hepatic immaturity of the developing fetus, ethanol reaching the fetus from the maternal diet is metabolized into acetaldehyde only in the fetus brain by catalases, and no peripheral acetaldehyde is supposedly

produced by the hepatic alcohol dehydrogenase enzymes. In addition, it is important to recall that the acetaldehyde produced in the mother's liver does not cross the placenta (at least with the moderate ethanol doses used here). In sum, the only acetaldehyde experienced by the fetus after prenatal ethanol administration to the mother is that produced in its brain by the catalase system. Interestingly, this is the central acetaldehyde that has been shown to have reinforcing effects in adult and neonate rats (Karahanian et al., 2011; Quertemont & Tambour, 2004; March et al, 2012; Pautassi et al, 2011). Given the fact that in our studies acetaldehyde was not directly administered, but was derived from ethanol, the possibility exists that the sequestering drug would eliminate acetaldehyde, whilst ethanol would still be present in the amniotic fluid and the fetus' body for a longer time until its complete metabolization and/or elimination. In this highly probable case, the complete absence of a postnatal response to ethanol flavor (no increased acceptance at any age) observed in our experiments, may indicate that acetaldehyde is the main, if not the only, prenatal reinforcer responsible for the effect studied in this thesis. However, this needs to be further investigated by directly manipulating the presence of either substance (ethanol or acetaldehyde) possibly through the enzymes involved in each step of the ethanol metabolic chain. In addition, further studies are necessary to investigate the connection between prenatal acetaldehyde and the stimulation of the fetal opioid system, which undoubtedly

mediates the reinforcing effects of ethanol in both infancy and prenatal stages. In conclusion, this set of findings constitutes the first steps of a promising line of enquiry to determine the role played by each of the elements involved in the chain between prenatal ethanol exposure and the increased acceptance of ethanol observed at different postnatal stages.

CONCLUSIONS



Taken together, the results of all the experimental work described in this thesis prompt the following conclusions

1. Prenatal experience with a non-ethanol flavor without pharmacological effects may result in an increased attraction for this flavor, an effect that is detected soon after birth. This may be taken to support the notion of a role for the KIF component of the amniotic fluid as the reinforcer in a prenatally acquired appetitive response. However, this reinforcer appears to be rather weak, given that the learned response is not retained for a relatively long period, even when using techniques more suitable for measuring behavior learned at this developmental stage
2. The activation of the mu-opioid receptor system appears to be critical, not only for prenatal appetitive learning about ethanol, but also for learning about other non-ethanol flavors. However, kappa-opioid receptors seem to participate only in the prenatally learned response to ethanol.
3. Acetaldehyde, rather than ethanol, seems to be the chief reinforcer involved in prenatal appetitive learning about the ethanol flavor.

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