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Reward sensitivity, social motivation and endogenous opioid signaling in adolescent C57BL/6 mice

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Scan the QR code to access the **full PDF** of my PhD thesis and a **simplified audiobook** version.

The audiobook covers sections:
3 (Abstract), 4 (Introduction), 5 (Research objectives), 6.2 (Additional unpublished research results), and 7 (Discussion), without figure descriptions or citations.

This PhD has taught me that

Mistakes will always happen - what truly matters is recognizing them,
learning from them, and moving forward.

Science is a long run, not a sprint. You have to keep your pace and care for your energy,
or you'll burn out before reaching the finish line.

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List of scientific articles constituting the basis of the doctoral thesis

Original paper 1

Misiołek K, Klimczak M, Chrószcz M, Szumiec Ł, Bryksa A, Przyborowicz K, Rodriguez Parkitna J, (2023) Harda Z. *Prosocial behavior, social reward and affective state discrimination in adult male and female mice.* Sci Rep, 13, 5583, doi: 10.1038/s41598-023-32682-6

Original paper 2

Misiołek K, Chrószcz M, Klimczak M, Rzeszut A, Netczuk J, Ziółkowska B, Szumiec Ł, Kaczmarczyk-Jarosz M, Harda Z, & Rodriguez Parkitna J (2025) *Adolescent mice exhibit lower reward sensitivity than adults.* Front. Behav. Neurosci., 19. doi: 10.3389/fnbeh.2025.1695375

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1. List of abbreviations

ANOVA – Analysis of variance

BOTSA – Brain Opioid Theory of Social Attachment

cDNA – Complementary DNA

CPA – Conditioned place aversion

CPP – Conditioned place preference

Ct - Cycle threshold

D1 – Dopamine receptor 1

Drd1 **-** Dopamine receptor D1 gene

D2 – Dopamine receptor 2

Drd2 – Dopamine receptor D2 gene

DAPI – 4′,6-diamidino-2-phenylindole

DOR – δ opioid receptor

Oprd1 – δ opioid receptor gene

DS – Dorsal striatum

E-ado – Early adolescence, Pubertal onset (~P32)

GABA - gamma-aminobutyric acid

HPA – Hypothalamic-pituitary-adrenal axis

i.p. – Intraperitoneal

KOR – κ opioid receptor

Oprk1 –κ opioid receptor gene

L-ado – Late adolescence, sexual maturity (~P43)

MDMA – 3,4-methylenedioxymethamphetamine

M-ado – Mid-adolescence, peripubertal (~P38)

MOR – μ opioid receptor

Oprm1 **–** μ opioid receptor gene

mPFC – Medial prefrontal cortex

NAc – Nucleus accumbens

norBNI – nor-binaltorphimine (selective KOR antagonist)

P32 / P38 / P43 – Postnatal day references for developmental stages

DYN –dynorphin

Pdyn – Prodynorphin gene (precursor to dynorphin peptides)

Penk – Proenkephalin gene (precursor to enkephalin peptides)

qPCR – Quantitative polymerase chain reaction

ROI – Region of interest

RT - Room temperature

SEM – Standard error of the mean

Slc32a1 – Vesicular GABA transporter gene

Sst – Somatostatin gene

2. Streszczenie

Zachowania społeczne ułatwiają zdobywanie zasobów, pozwalają na unikanie zagrożeń i sprzyjają reprodukcji. Interakcje społeczne są nagradzające, a zmiany w ich wartości motywacyjnej w trakcie rozwoju odzwierciedlają dojrzewanie układów neuronalnych regulujących nagrodę, uczenie się i stres. W mojej pracy doktorskiej zbadałam, jak zmieniają się nagradzające właściwości kontaktów społecznych w kontekście niereprodukcyjnym u myszy C57BL/6 na różnych etapach adolescencji: wczesnej (początek dojrzewania), środkowej (okres dojrzewania) i późnej (osiągnięta dojrzałość płciowa). Moje wyniki wskazują, że kluczowym mechanizmem kształtującym dojrzewanie zachowań społecznych mogą być zmiany w endogennej sygnalizacji opioidowej.

Na początku zbadałam zachowania prospołeczne, zdolność rozpoznawania stanów afektywnych oraz warunkowaną kontekstem społecznym preferencję miejsca (CPP) u dorosłych myszy. Samice częściej podejmowały wybory prospołeczne niż samce, natomiast płeć nie wpływała na rozpoznawanie stanu afektywnego innego osobnika ani na preferencję kontekstu skojarzonego z interakcją społeczną. Dorosłe myszy były wrażliwe na złożone bodźce społeczne, co potwierdza ich przydatność jako modelu do badań neuronalnych mechanizmów motywacji społecznej.

Następnie przeanalizowałam, jak wrażliwość na nagradzające efekty kontaktów z rówieśnikami zmienia się w okresie dojrzewania. Zarówno samce, jak i samice w fazie adolescencji wykazywały słabszą preferencję kontekstu społecznego niż osobniki dorosłe. Zaobserwowałam również osłabioną preferencję dla smacznego pożywienia, podczas gdy preferencja dla nagrody kokainowej pozostawała porównywalna z dorosłymi. Analiza łącząca wyniki wszystkich typów nagród wykazała, że dojrzewające myszy wykazują niższą ekspresję CPP, co stoi w sprzeczności z poglądem, że okres dojrzewania wiąże się ze zwiększoną wrażliwością na nagrody.

Wcześniejsze badania wskazały na istotną rolę endogennych opioidów w regulacji zachowań społecznych. W moich badaniach przeanalizowałam zmiany w ekspresji genów kodujących receptory i peptydy opioidowe w różnych etapach adolescencji. U myszy w późnym okresie dojrzewania zaobserwowałam obniżenie ekspresji prodynorfiny (*Pdyn*) w przyśrodkowej korze przedczołowej i

grzbietowym prążkowiu, a jednocześnie wzrost ułamka komórek wykazujących ko-ekspresję *Pdyn* z mRNA somatostatyny w korze przedczołowej lub z mRNA receptora opioidowego κ (KOR) w prążkowiu i jądrze półleżącym.

Aby sprawdzić, czy zmiany te mają znaczenie funkcjonalne, przeprowadziłam eksperymenty z wykorzystaniem CPP przy farmakologicznym lub genetycznym zahamowaniu działania KOR. Zarówno podanie antagonisty KOR (norbinaltorfiminy), jak i całkowita inaktywacja genu *Pdyn* selektywnie osłabiały społeczne, ale nie kokainowe CPP - i to wyłącznie w późnej adolescencji. Wyniki te sugerują, że w okresie dojrzewania motywacja społeczna ulega jakościowym zmianom: młodsze osobniki są mniej zależne od obwodów związanych ze stresem, podczas gdy starsze coraz bardziej angażują szlaki dynorfina/KOR modulujące reakcje stresowe.

Moja praca, łącząc dane behawioralne, genetyczne i farmakologiczne, wskazuje na rolę układu dynorfina/KOR w rozwoju nagradzających właściwości interakcji społecznych w kontekście niereprodukcyjnym. Jako pierwsza opisałam zmiany w ekspresji genów opioidowych w przyśrodkowej korze przedczołowej i prążkowiu u myszy w okresie adolescencji. Uzyskane wyniki poszerzają obecną wiedzę, sugerując, że dojrzewanie układu dynorfina/KOR może odpowiadać za ulgę związaną z przerwaniem izolacji społecznej, co wskazuje na potencjalne powiązanie między motywacją społeczną w okresie dojrzewania a obwodami neuronalnymi wrażliwymi na stres.

3. Abstract

Social behaviors improve access to essential resources, reduce vulnerability to threats, and provide reproductive opportunities. They are inherently rewarding, and the motivational value of social interactions changes across adolescence, likely reflecting the maturation of neural systems regulating reward, learning, and stress. Here, I investigated the rewarding effects of social contact in a non-reproductive context in early (pubertal onset), mid (peripubertal), and late (sexual maturation) adolescent C57BL/6 mice. I show that changes in endogenous opioid signaling may be a key mechanism shaping the maturation of social behavior.

First, I assessed prosocial behavior, affective state discrimination, and social conditioned place preference (CPP) in adult mice. Females were significantly more likely to perform prosocial choices than males. However, sex had no effect on the ability to discriminate the familiar conspecific with a changed affective state, or on preference for a context associated with sibling social housing. Adult mice were highly responsive to social cues, establishing a robust model for examining the neural mechanisms of social motivation.

Next, I examined how adolescence shaped sensitivity to the rewarding effects of social interaction. Both male and female adolescent mice displayed a lower preference for a context associated with social housing than adults. Adolescents also showed diminished palatable food CPP, suggesting a general decrease in natural reward preference, while sensitivity to cocaine was comparable to that of adults. When data across all reward types were analyzed together, adolescence was consistently associated with lower overall CPP expression, challenging the view of adolescence as a period of increased reward sensitivity.

Previous work has pointed to endogenous opioid signaling as a critical modulator of social behavior. I analyzed developmental changes in the expression of genes encoding opioid receptors and peptides. Across adolescence, prodynorphin (*Pdyn*) expression decreased in the medial prefrontal cortex and dorsal striatum, while the proportion of cells co-expressing *Pdyn* with somatostatin mRNA in the medial

prefrontal cortex, or with κ opioid receptor (KOR) mRNA in the dorsal striatum and nucleus accumbens, increased.

To test whether these developmental shifts in basal *Pdyn* expression are associated with behavioral changes, I assessed social and cocaine CPP following pharmacological or genetic inhibition of KOR function. Administration of the long-acting KOR antagonist - norbinaltorphimine, as well as complete *Pdyn* gene inactivation, selectively reduced social, but not cocaine CPP, only in late adolescence. These findings suggest that social motivation undergoes qualitative changes across adolescence, with younger stages less dependent on stress-sensitive circuits and more mature stages increasingly recruiting stress-modulating dynorphin/KOR pathways.

By integrating behavioral, genetic, and pharmacological evidence, my work demonstrates how the dynorphin/KOR system contributes to the maturation of social reward in non-reproductive contexts. I provide the first characterization of adolescent reorganization of opioid gene expression in the medial prefrontal cortex and striatum, revealing age-dependent refinements in dynorphin-expressing neuronal populations. These findings advance current knowledge by suggesting that developmental refinement of the dynorphin/KOR system may increase the role of relief from isolation in peer social drive, offering a potential link between adolescent social motivation and stress-sensitive neural circuits.

4. Introduction

Rewards lie at the core of what drives behavior (Schultz, 2015). They motivate organisms to eat, drink, and reproduce - behaviors essential for survival. The brain, and particularly the reward system, evolved to guide behavior, allowing organisms to navigate through their environment and learn which experiences lead to the best outcomes. Through learning and decision-making, the brain identifies what is valuable, pursues it effectively, and produces satisfaction when the goal is achieved. As Wolfram Schultz emphasized, rewards are defined not by their physical properties, but by the behaviors and internal states they evoke (Schultz, 2015). Therefore, understanding reward requires theoretical and behavioral frameworks that connect subjective experience to measurable behavior. Studying these foundations helps explain how the brain transforms motivation into adaptive action.

Building on understanding of how the brain processes rewards, my research focuses on the neural mechanisms underlying social behavior. Social interaction represents a uniquely powerful form of reward, relying on the brain's ability to interpret others' mental states, integrate sensory and emotional information, and produce contextually appropriate responses. Much like reward processing, the field of social neuroscience demonstrates how deeply our brains are attuned to social connection: to detect, interpret, and respond to social cues (Stanley & Adolphs, 2013). This PhD thesis explores the intersection between reward and social processing, uncovering the neurobiological mechanisms through which the brain transforms internal drives into meaningful social behavior.

4.1 Opioids and social reward

Social behavior, defined broadly as any form of physical interaction or non-physical communication between members of the same species, represents one of the most powerful drivers of behavior across mammals (Rubenstein & Rubenstein, 2013). It encompasses diverse interactions, including mating, parental care, aggression, and affiliation (Silk, 2007; Chen & Hong, 2018). Aggressive behaviors establish dominance hierarchies and regulate access to resources, while affiliative behaviors, such as grooming and cooperation, promote cohesion and reduce conflict. Social structures emerge from

ecological pressures such as resource distribution, predation risk, and habitat organization, which shape the costs and benefits of group living. Typically, females organize around ecological challenges like foraging and offspring care, while males track female distributions, giving rise to mating systems ranging from monogamy to polygyny and cooperative breeding (Rubenstein & Rubenstein, 2013). Beyond these well-studied behaviors, non-reproductive affiliative relationships, which could colloquially be referred to as *friendships*, play a critical role in group stability and individual well-being (Clark & Dumas, 2015). Many animals form preferential bonds with close kin or with peers of similar age, social status, and behavioral traits (Chapais & Berman, 2004; Brent *et al.*, 2014).

Friendship-like relationships serve as a unique form of social affiliation, which constitutes the primary context for everyday social experiences, shaping mood, decision-making, and emotional and cognitive health. Their adaptive value lies in providing frequent, low-risk opportunities to practice social behaviors, refine cooperative strategies, and navigate social rules, making these interactions a natural context for social learning: individuals observe, imitate, and adapt behaviors through social reinforcement (Bandura, 1971). Through these exchanges, behavioral norms are transmitted, group cohesion is strengthened, and cooperative success is enhanced, fulfilling both informational and emotional functions. Clinical evidence further illustrates that peer interactions strengthen coping skills, social functioning, and emotional regulation. For instance, peer-support interventions leverage social affiliation to promote recovery, empowerment, and social integration across clinical populations (Shalaby & Agyapong, 2020). These observations exemplify that peer-social interactions are fundamental to survival, cooperation, and collective fitness (Brent *et al.*, 2014).

Much like physiological drives such as hunger or sleep, social needs are homeostatically regulated (Liu *et al.*, 2025). In humans, altered social responsiveness and disrupted social motivation characterize several neuropsychiatric disorders, including autism spectrum disorder, schizophrenia, and psychopathy, all of which involve dysfunction in social reward processing (*DSM-5*, 2013). In mice, unmet social needs have profound consequences: social deprivation causes long-term social, cognitive, emotional and metabolic impairments (Hu *et al.*, 2023; Huang *et al.*, 2024; Geng *et al.*, 2025). Thus, maintaining social connectedness is not merely advantageous but biologically essential, supporting

adaptive behavior and mental health. Despite their importance, the neurobiological mechanisms underlying the formation and maintenance of *peer relationships* remain only partly understood. Because these relationships are sustained by reward and reinforcement, processes collectively referred to as social reward, a central question emerges: *what are the neural circuits that mediate peer-social reward?*

Social-seeking behaviors are evolutionarily conserved across mammals, pointing to shared neural substrates. In humans, infants spend more time looking at faces than at non-face stimuli (Johnson *et al.*, 1991). Similarly, in mice, the presence of social motivational drive is evidenced by the preference to interact with conspecifics rather than with inanimate objects (Moy *et al.*, 2004). The "social brain" comprises a network of corticolimbic regions encoding the reward value of social signals, including the medial prefrontal cortex (mPFC), the dorsal striatum (DS), the nucleus accumbens (NAc), the amygdala, and the ventral tegmental area (VTA) (Fernández *et al.*, 2018; Grimm *et al.*, 2021). The mPFC supports decision-making, behavioral flexibility, response inhibition, attention, and emotional regulation, key components of adaptive social behavior. The amygdala contributes to stress regulation and emotional processing of social stimuli, while structures such as the NAc and VTA are central to the rewarding aspects of social interaction. The DS, typically associated with habit formation, also participates in motivation and partner preference formation (Báez-Mendoza & Schultz, 2013). Within these circuits, multiple neurotransmitter and neuropeptide systems, including glutamate, gamma-aminobutyric acid (GABA), oxytocin, serotonin, and dopamine, interact to modulate social motivation, memory, and affective responses (Dölen *et al.*, 2013; Fernández *et al.*, 2018; Scheggia *et al.*, 2020; Li *et al.*, 2025). The coordinated activity of these systems underlies multiple aspects of social behavior; however, the present thesis focuses specifically on social reward - the affective and motivational processes driving peer-directed interactions.

Social reward can be conceptualized within the same motivational framework that governs reward processing in general (Berridge *et al.*, 2009; Grimm *et al.*, 2021). It underlies the decisions to seek, maintain, or avoid social interactions and refers to the intrinsic pleasurable impact of social contact mediated by "hedonic hotspots" distributed across the brain (Berridge *et al.*, 2009). Social motivation, the drive to approach social contact, arises from mesolimbic dopamine activity encoding the salience of

social stimuli; an increase in dopamine signaling increases motivation to approach social cues. Together, social reward ("liking") and social motivation ("wanting") form a motivational system promoting engagement with social stimuli (Grimm *et al.*, 2021). Complementing dopamine-driven motivation, the opioid system mediates the hedonic and emotional component of social reward, or "liking" (Merrer *et al.*, 2009; Fields & Margolis, 2015). This system consists of endogenous peptides (enkephalins, dynorphins, and β-endorphin) and their receptors μ (MOR), κ (KOR), and δ (DOR), which are ubiquitously expressed across the brain (Merrer *et al.*, 2009). The opioid system modulates multiple neuronal populations, including dopamine, GABA, and glutamate neurons, primarily producing inhibitory effects that can lead to either inhibition or disinhibition of activity depending on the neural circuit context. By signaling reward, reducing negative affect and in certain contexts, inducing aversion or modulating stress responses - the opioid system shapes social behavior in ways that extend beyond simple reinforcement and can operate independently of dopaminergic signaling (Fields & Margolis, 2015). This complementary interplay between dopamine and endogenous opioids provides a neurobiological basis for the processes that make social interactions rewarding and motivating.

The significance of the opioid system in social behavior is emphasized in the Brain Opioid Theory of Social Attachment (BOTSA) proposed by Panksepp (Panksepp *et al.*, 1980). BOTSA posits that endogenous opioids, particularly endorphins, play a central role in modulating social behavior and emotional states. These systems enhance the rewarding aspects of social interactions while mitigating distress associated with social isolation, particularly in juveniles (Herman & Panksepp, 1978). It was observed that opioid agonists, such as morphine, increase social play, dominance behaviors, and persistence in socially motivated tasks, whereas opioid antagonists, like naloxone, reduce these behaviors and can induce anxiety or distress (Herman & Panksepp, 1978; Panksepp *et al.*, 1980, 1985; Vanderschuren, Niesink, *et al.*, 1995; Vanderschuren, Spruijt, *et al.*, 1995; Trezza *et al.*, 2011). The early attachment process is supported by opioid-mediated reinforcement, as social contact provides both comfort and positive affect. Furthermore, social deprivation alters opioid receptor density and increases voluntary opiate intake, suggesting an adaptive role of these systems in maintaining social homeostasis (DeFeudis, 1978; Panksepp *et al.*, 1980). Dysregulation of opioid activity contributes to psychiatric

disorders, indicating the importance of balanced opioid function for normative social and emotional development (Panksepp, 1979; Peciña *et al.*, 2019; Charles *et al.*, 2020). Together, these findings support the BOTSA, illustrating how endogenous opioids regulate social reward and attachment, and highlighting neural processes directly relevant to the mechanisms of peer-directed social behavior investigated in this thesis.

MOR, DOR, and KOR play distinct and sometimes opposing roles in social behavior (Merrer *et al.*, 2009; Inagaki, 2018; Galiza Soares *et al.*, 2025). MORs in regions like the VTA, NAc, PFC, amygdala or habenula generally promote positive social interactions (Merrer *et al.*, 2009; Galiza Soares *et al.*, 2025). For example, MOR activation in the VTA disinhibits dopaminergic neurons, increasing dopamine release in the NAc to increase social reward, while MORs in the NAc directly enhance social play and approach behaviors. In contrast, KORs in the NAc, PFC, VTA, amygdala, and hypothalamus drive social avoidance and stress-induced social withdrawal by reducing dopamine and serotonin release, potentiating dysphoria, and strengthening "anti-reward" circuits (Merrer *et al.*, 2009; Galiza Soares *et al.*, 2025). DORs, particularly in the NAc, DS, or PFC, often interacting with MOR and KOR circuits, appear to modulate motivational salience of social cues, though their role is less clearly defined. Translating these findings to humans, evidence shows that blocking MORs reduces feelings of warmth and connection during positive interactions with close others, while stimulating opioid activity enhances these positive feelings and can also reduce distress during socially stressful situations (Inagaki, 2018). How opioids influence social behavior depends on the type of social experience and the closeness of the relationship, and this sensitivity can be shaped by early life experiences. For example, social adversity such as isolation can alter the development of opioid and monoamine circuits, producing lasting changes in how social interactions are experienced and how these systems respond in adulthood (Galiza Soares *et al.*, 2025). Together, these findings indicate that MOR, DOR, and KOR operate within a network that balances social reward and aversion, shaping how individuals experience and respond to social interactions.

Thus, dopamine, opioids, oxytocin, and serotonin form a neuromodulatory system that coordinates approach and avoidance in social behavior. Among these, the opioid system plays a central role in

integrating hedonic and reward-related components of social interaction. While dopaminergic mechanisms of social drive have received a lot of attention, the contribution of endogenous opioids remains relatively underexplored. This thesis, therefore, focuses on the opioid system to understand how neural reward mechanisms support the pursuit, maintenance, and enjoyment of peer-directed social interactions.

4.2 Animal models of social reward

Laboratory rodents, including the C57BL/6 mice, are highly social animals that display a broad repertoire of social behaviors as well as social deficits similar to those observed in human psychiatric disorders (Crawley, 2007; Matsuo *et al.*, 2010; Netser *et al.*, 2020). These features, along with well-characterized genetics and conserved neurobiological pathways, make mice a useful model for dissecting the molecular and circuit-level mechanisms of social behavior (Fernández *et al.*, 2018).

To quantify social reward in mice, researchers usually employ the conditioned place preference (CPP) paradigm (Panksepp & Lahvis, 2007; Dölen *et al.*, 2013; Nardou *et al.*, 2019). In the CPP task, animals learn to associate an initially neutral context with a stimulus, and the expression of preference for that context after conditioning indicates the stimulus has rewarding properties (Rossi & Reid, 1976; Cunningham *et al.*, 1997; Tzschentke, 1998; Bardo & Bevins, 2000; Prus *et al.*, 2009). This paradigm integrates reinforcement, associative learning, conditioned responses, and motivational drive (McKendrick & Graziane, 2020). Motivation reflects the organism's internal state, integrating external cues and internal signals to coordinate adaptive responses (Flavell *et al.*, 2022). CPP reflects the balance between two opposing motivational processes: positive reinforcement, representing the intrinsic rewarding properties of the unconditioned stimulus, and negative reinforcement, reflecting aversive or stress-related states (Rossi & Reid, 1976; Tzschentke, 1998; Bardo & Bevins, 2000; Navratilova *et al.*, 2013; McKendrick & Graziane, 2020). The resulting approach or avoidance behavior depends on the balance between these processes.

Drug-induced CPP paradigms, well-established in classical pharmacology, provide a framework for understanding the mechanisms of reward learning and reinforcement. These studies show how positive

and negative reinforcement shape context-reward associations, offering a benchmark for interpreting social CPP. In this regard, the work of Ettenberg and colleagues demonstrated that cocaine induces CPP when context pairing occurs immediately after injection but produces conditioned place aversion when a 15-minute delay is introduced before exposure to the context (Ettenberg *et al.*, 1999). This phenomenon reflects cocaine's biphasic motivational profile, an initial dopaminergic surge associated with euphoria, followed by a dysphoric "crash" (Carlezon & Thomas, 2009; Koob & Volkow, 2010). Pairing a context with drug exposure during this aversive phase can promote CPP through negative reinforcement, that is, the learned relief of a dysphoric or stress-related state. Similar biphasic motivational dynamics have been reported for other psychostimulants and sedatives, including ethanol, nicotine, and amphetamine, emphasizing that reward involves both positive rewarding and negative relief-based reinforcement mechanisms (Fudala & Iwamoto, 1987, 1990; Cunningham *et al.*, 1997). Building on these principles, the CPP paradigm has been adapted to study social reward. The version used in this PhD thesis was first developed by Panksepp and Lahvis (Panksepp & Lahvis, 2007) to examine social reward in juvenile mice and has since been used to explore its underlying neurobiology (Dölen *et al.*, 2013; Nardou *et al.*, 2019; Cann *et al.*, 2020). In social CPP, animals learn to associate one environment with group housing and another with isolation. The increased time in the social-paired environment reflects the rewarding value of peer interactions in a non-reproductive context. Panksepp and Lahvis demonstrated that juvenile mice from the A, B6, and DBA strains develop a strong preference for environments associated with social contact, while BALB mice show little interest in social interaction but respond normally to food rewards. This social preference does not depend on the sex of cage mates, maternal care, or litter size, but it emerges only after a period of social isolation, suggesting that social CPP reflects possibly both the pleasurable effects of reuniting with peers and the aversion to being alone (Panksepp & Lahvis, 2007).

Subsequent work elucidated the neurobiological basis of social reward. Dölen and collaborators showed that oxytocin and serotonin cooperate to encode social motivation: oxytocin, released from the hypothalamus, acts on presynaptic terminals in the NAc to induce long-term depression of excitatory inputs, a process that depends on serotonin receptor 1b signaling from the dorsal raphe nucleus (Dölen

et al., 2013). Disrupting either pathway abolishes social CPP, revealing a mechanistic basis for social reward that may be relevant to altered social behavior observed in autism spectrum disorder (Dölen *et al.*, 2013). Nardou and colleagues expanded this work by identifying a critical period for social reward learning in juvenile mice, during which oxytocin-dependent synaptic plasticity in the NAc reaches a peak. The window for social reward learning closes before adulthood but can be pharmacologically reopened by the drug 3,4-methylenedioxymethamphetamine (MDMA), which enhances oxytocin-dependent long-term depression of neuronal activity. Restoration of social CPP in adult mice required both oxytocin receptor activation and an appropriate social context, providing a potential mechanism through which MDMA could support social learning in therapeutic settings, such as post-traumatic stress disorder (Nardou *et al.*, 2019). Further work by the same group demonstrated that other psychedelics, including lysergic acid diethylamide, psilocybin, ketamine, and ibogaine, are also capable of reopening this social reward learning critical period in adult mice (Nardou *et al.*, 2023). In parallel, Cann and collaborators investigated the factors that influence social CPP in C57BL/6J mice, including age, sex, prior social experience, and environmental cues. Their results showed that social CPP is highly context-dependent: early adolescent males exhibited a temporary preference for social cues only when housed in groups before testing, while females required a five-day period of social isolation to display a similar preference. Additionally, social CPP was short-lived and sensitive to procedural variables, in contrast to the strong and persistent place preference produced by cocaine. These findings highlight the variability of social CPP and the importance of careful experimental design when studying the neural mechanisms underlying social reward (Cann *et al.*, 2020).

Adult C57BL/6 mice, despite their sociability and sensitivity to isolation stress, were reported to show no significant social CPP (Nardou *et al.*, 2019, 2023). During my PhD project, our team observed that adult females developed social CPP when the conditioning period was extended, allowing more cue-context associations, and when the social partner was a sibling, indicating the influence of kinship on peer social reward (Harda *et al.*, 2022). These findings suggest that adult social reward expression is not absent but rather becomes apparent only under specific experimental conditions. However, it remained unclear whether adult males show similar social CPP. Notably, in the wild, adult male and female *Mus*

musculus exhibit distinct social strategies: males are typically more territorial and competitive (Groó *et al.*, 2013; Kondrakiewicz *et al.*, 2019) while females engage in cooperative behaviors such as communal nesting and nursing (Hayes, 2000). Both affiliative and agonistic interactions can be rewarding depending on context (Golden *et al.*, 2019; Aubry *et al.*, 2022), which suggests that "social reward" may not represent a unitary process but rather a constellation of motivational drives modulated by sex, dominance hierarchies, and previous experience. Thus, at the start of my experiments, it was still unclear whether adult male and female mice express social CPP and how their distinct social strategies might influence this behavior.

Ultimately, studying peer-social reward requires behavioral paradigms that capture its multidimensional and context-dependent nature. In mice, oxytocin and serotonin signaling in the NAc mediate core mechanisms of social motivation, but social CPP likely integrates both the positive reinforcement of social contact and the relief from isolation, indicating contributions from additional neural circuits. Robust social CPP is consistently observed in juveniles, but responses in adults are weaker and more variable. Whether this reflects developmental differences in social reward or arises from experimental conditions remains a critical question. Resolving this issue is essential for understanding the ontogeny of non-reproductive social motivation and for improving the design and translational relevance of social reward research.

4.3 Maturation of the reward system during adolescence

Adolescence is a critical transitional period, representing the final stage of extensive brain maturation. During this developmental phase, neural circuits supporting emotion, reward, cognition, and social behavior exhibit enhanced plasticity, making adolescence both a window for last-minute adaptive changes and a vulnerable period for maladaptive alterations (Spear, 2000; Knudsen, 2004; Dow-Edwards *et al.*, 2019; Uhlhaas *et al.*, 2023; Baker *et al.*, 2025). Early intervention during adolescence could thus help to prevent or mitigate long-term mental illness, whereas stress, trauma, or substance exposure at this time may trigger cascading effects that increase the risk of psychiatric disorders (Spear, 2000; Laviola *et al.*, 2003; Laviola & Marco, 2011; Sheth *et al.*, 2017; Uhlhaas *et al.*, 2023).

Adolescence is marked by pronounced behavioral shifts, including increased reward, novelty and sensation seeking (Adriani *et al.*, 1998; Spear, 2000; Laviola *et al.*, 2002; Knudsen, 2004; Galván, 2010; Wahlstrom *et al.*, 2010; Vetter-O'Hagen & Spear, 2012), greater risk-taking (Laviola *et al.*, 2003; de Water *et al.*, 2014; Weisfeld & Shattuck, 2017), and an elevated susceptibility to drugs of abuse (Schramm-Sapyta *et al.*, 2009; Arguello *et al.*, 2024). Adolescents are particularly sensitive to social context and respond to social rewards differently than adults, showing an increasing desire for autonomy from parents, stronger peer influence, exploration of identity, questioning of adult norms, greater sensitivity to social status, and experimentation with social roles (Spear, 2000; Furstenberg, 2001; Doremus-Fitzwater *et al.*, 2010; Spear, 2013). Together, these behavioral changes support a shift in social interest from family members to non-kin peers (Spear, 2000, 2013). Similarly, in rodents, a decrease in kin-directed social reward coincides with dispersal behaviors, a species-typical adaptation that reduces inbreeding and promotes outbreeding success (Panksepp & Lahvis, 2007; Lukas & Clutton-Brock, 2011; Lin & Wilbrecht, 2022). Early-life social behavior is dominated by kin-directed interactions, primarily involving parents. During adolescence, these interactions gradually give way to peer- and non-kin-directed behaviors. As affiliative play and grooming decrease, the dominance, territoriality, and sexually motivated behaviors increase (Panksepp *et al.*, 2007; Varlinskaya & Spear, 2008; Pellis & Pellis, 2017). This shift reflects the ongoing reorganization of social circuits, highlighting adolescence as a critical period for studying the neural mechanisms of peer-directed social reward.

The neurobiological underpinnings of these behavioral changes are closely tied to the maturation of the brain's reward system (**Figure 1A**). The mesolimbic and mesocortical dopamine pathways undergo extensive structural and functional changes that support the development of the target regions - striatum and PFC (**Figure 1B**). In the PFC, dopamine fibers continue to grow and form synapses during adolescence, and dopamine levels increase, supporting local circuit maturation (Hoops *et al.*, 2018; Reynolds & Flores, 2021). Dopamine receptor levels on pyramidal neurons and interneurons peak, enhancing dopamine signaling in both cell types; these changes help fine-tune the balance between excitation and inhibition in the PFC, supporting the development of mature cognitive control (Brenhouse *et al.*, 2008; Reynolds & Flores, 2021; Avramescu *et al.*, 2024). In the striatum, dopamine content rises

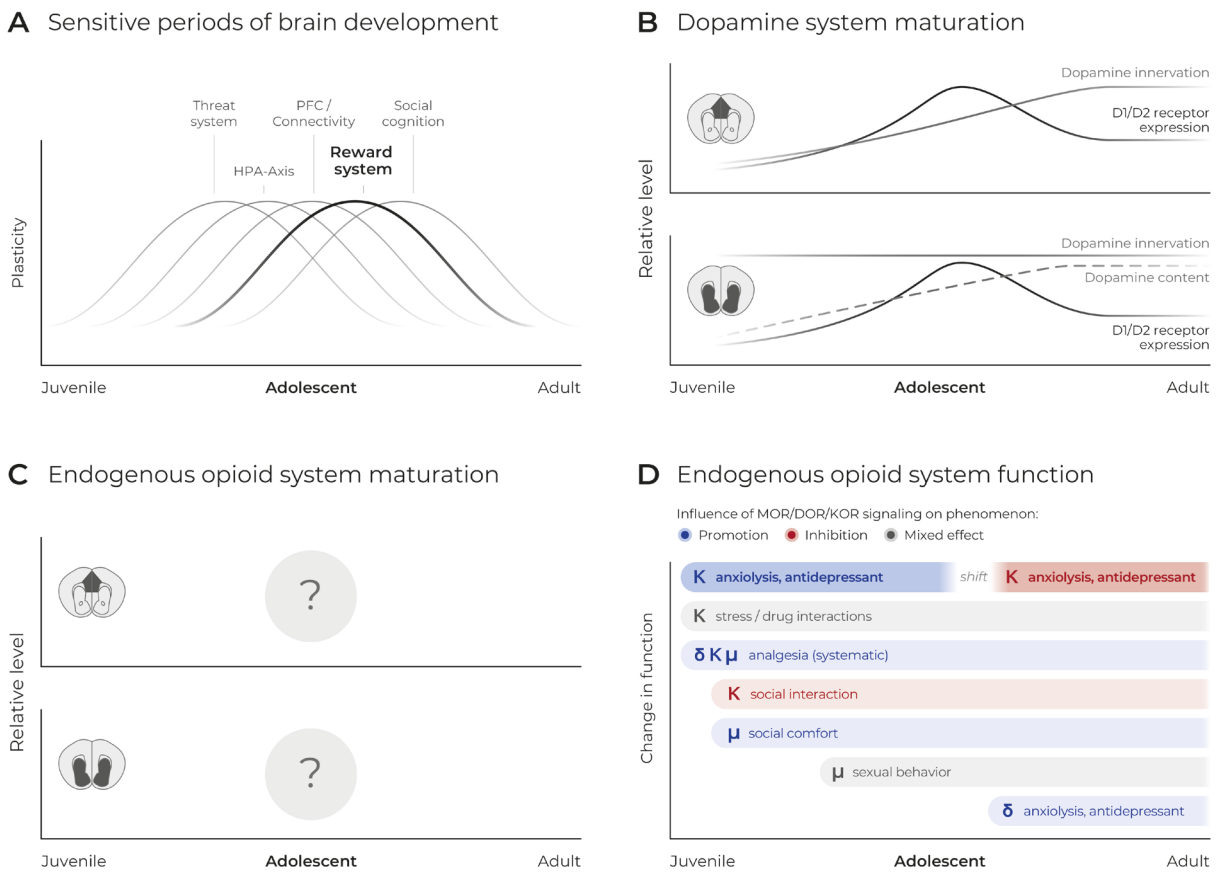

Figure 1. Adolescent changes in neural plasticity and corresponding adaptations in dopamine and opioid systems.

(A) Sensitive periods during brain development (adapted and modified from Uhlhaas *et al.*, 2023). Curves represent the relative plastic potential of different neural systems, peaking during adolescence: threat regulation involving cortical–hippocampal–amygdala circuits, the hypothalamic–pituitary–adrenal (HPA) axis, prefrontal cortex (PFC) local and long-range connectivity with cortical and subcortical targets, the reward system including striatal circuits and social behavior networks.

(B) Adolescent maturation of dopamine connectivity and function in the PFC (top) and striatum (bottom) (adapted and modified from Reynolds & Flores, 2021). Curves indicate developmental changes in the dopamine system, including peak dopamine receptor 1 (D1) and dopamine receptor 2 (D2) expression, pruning of dopaminergic innervation, and increases in dopamine content.

(C) Data on adolescent maturation of opioid gene expression and function in the PFC (top) and striatum (bottom) are currently lacking.

(D) Summary of preclinical studies illustrating the role of opioid signaling across development (adapted and modified from Spodnick *et al.*, 2025). Receptors are positioned along the timeline according to the developmental periods they influence. Blue text indicates promotion of a developmental process by opioid signaling; red text indicates inhibition; gray text indicates complex or context-dependent effects. μ – MOR, κ- KOR, δ- DOR

alongside structural changes in medium spiny neurons, including increased spine density and refined excitability. D1 and D2 receptors are transiently overexpressed and then pruned, in a sex-specific manner (Andersen *et al.*, 1997; Reynolds & Flores, 2021; Avramescu *et al.*, 2024).

Adolescence is regarded as a period of heightened reward sensitivity, which is thought to be driven primarily by the dopamine-rich striatum, particularly the NAc (Spear, 2000; Knudsen, 2004; Dow-Edwards *et al.*, 2019; Uhlhaas *et al.*, 2023; Baker *et al.*, 2025). However, the nature of adolescent reward sensitivity is debated. Some evidence suggests hypo-responsiveness, with lower basal dopaminergic activity requiring stronger or more frequent rewards, consistent with increased negative affect and reduced enjoyment of primary reinforcers like sugar, whereas other findings support hyper-responsiveness, with exaggerated dopaminergic responses enhancing motivation for high-reward behavior (Panksepp, 1998; Galván, 2010). Overall, while hyper-responsiveness is often emphasized in both animal and human studies, striatal reward processing during adolescence likely reflects a dynamic and context-dependent balance, rather than uniformly heightened sensitivity (Spear, 2000; Galván, 2010; Spear, 2013).

Among the neural systems that mature during adolescence, the endogenous opioid system is particularly important, as it shapes both reward processing and social motivation. However, the developmental trajectory of opioid receptor gene expression in the PFC and striatum remains poorly characterized, and its contribution to age-related changes in social reward is not fully understood (**Figure 1C**). Most studies have focused on prenatal, neonatal, early juvenile, or adult stages, leaving adolescence relatively unexplored (Thorpe *et al.*, 2020; Spodnick *et al.*, 2025). Preclinical evidence suggests that the functional roles of opioid receptors evolve across development: while MOR-, DOR-, and KOR-mediated analgesic effects are relatively stable from juvenile to adult stages, certain receptor-specific functions emerge specifically during adolescence (Spodnick *et al.*, 2025, **Figure 1D**). Notably, KOR exhibits pronounced developmental dynamics in this period, indicating it may play a key role in shaping age-specific behaviors (Anderson *et al.*, 2014; Varlinskaya *et al.*, 2018; Przybysz *et al.*, 2020). Therefore, the following section focuses on dynorphin (DYN)/KOR signaling, outlining its mechanisms and behavioral functions as a theoretical basis for the experimental approaches presented in this thesis.

4.4 DYN/KOR signaling

The DYN/KOR system, a key regulator of affective state and social motivation, is often described as an "anti-reward" pathway in the context of stress and drug withdrawal (Cayir *et al.*, 2024). It mediates the negative emotional consequences of stress, including social avoidance, depressive-like behavior, and increased vulnerability to drug seeking, largely via HPA axis activation (Hang *et al.*, 2015), which triggers corticotropin-releasing factor (CRF) signaling within limbic circuits (Land *et al.*, 2008). CRF2 receptor activation promotes DYN release and KOR stimulation in stress-responsive regions, including the basolateral amygdala, NAc, dorsal raphe nucleus, and hippocampus (Hang *et al.*, 2015). At the cellular level, acute KOR activation engages $G_{i/o}$ - coupled inhibition of adenylyl cyclase and recruits p38 MAPK signaling (Margolis *et al.*, 2003; Land *et al.*, 2008; Bruchas *et al.*, 2010). This cascade leads to suppression of mesolimbic dopaminergic signaling, producing effects such as dysphoria, anxiety-like behaviors, and conditioned place aversion (CPA).

The behavioral effects mediated by this system are dependent on multiple factors, including developmental stage and context. Selective KOR antagonists, such as nor-binaltorphimine (norBNI), restore normal reward processing rather than directly enhance reward (Jackson *et al.*, 2013; Khan *et al.*, 2022). By preventing stress-induced dopamine inhibition and long-term sensitization of corticolimbic circuits, a process in which repeated stress strengthens the responsiveness of these neural pathways, KOR antagonists restore social approach behavior and resilience to stress. Notably, in the absence of stress, KOR antagonism produces minimal change in social preference, demonstrating that DYN/KOR signaling primarily modulates the affective gating of social motivation rather than reward or aversion directly (Knoll & Carlezon, 2010; Jackson *et al.*, 2013).

KOR agonists, in contrast, can produce anxiogenic or anxiolytic effects depending on dose, brain region, timing, and developmental stage (Land *et al.*, 2008; Knoll & Carlezon, 2010; Hang *et al.*, 2015). These variable outcomes likely arise from the context-dependent modulatory role of DYN/KOR signaling, which integrates stress history, circuit activity, and local neurophysiology. For instance, acute or chronic stress or prior KOR activation suppresses NAc dopamine, manifesting as social anhedonia or reduced

peer engagement. On the other hand, mild or transient stress may temporarily elevate NAc dopamine, which is sometimes accompanied by activation of limbic circuits mediating negative valence, that can promote social avoidance (Land *et al.*, 2008; Bruchas *et al.*, 2010; Hang *et al.*, 2015). Prolonged KOR activation induces transcriptional and epigenetic adaptations within corticolimbic regions, including the PFC, NAc, and amygdala, enhancing sensitivity to future stress and sustaining negative affect (Knoll & Carlezon, 2010; Carlezon & Krystal, 2016). Moreover, repeated stress or drug exposure activates CREB, driving DYN upregulation in the NAc, which contributes to persistent dysphoric states. These adaptations can also potentiate dopaminergic responses to psychostimulants, illustrating stress-induced cross-sensitization. This process alters dopaminergic regulation of social reward, reducing sensitivity to social interactions and modifying social motivation. Acute DYN/KOR activation typically suppresses reward, whereas long-term adaptations can either exacerbate stress-induced dysphoria or enhance dopaminergic responses to rewards, depending on prior stress history and local circuit activity (Bruchas *et al.*, 2010; Knoll & Carlezon, 2010).

KOR signaling exhibits developmentally regulated effects, with its impact on affect and stress responsiveness changing from early life through adulthood. During the juvenile period and adolescence, KOR activation often produces anxiolytic and antidepressant-like effects, whereas in adulthood it typically becomes aversive, anxiogenic, and dysphoric (Land *et al.*, 2008; Braida *et al.*, 2009; Anderson *et al.*, 2014; Spodnick *et al.*, 2025). For example, low-dose KOR agonists do not influence or even reduce anxiety-like behaviors in young rodents, but elicit CPA and heightened anxiety in adults (Braida *et al.*, 2009; Anderson *et al.*, 2014; Przybysz *et al.*, 2020). During early adolescence, KOR activation enhances GABAergic inhibition in the basolateral amygdala, decreasing neuronal excitability and reducing anxiety, whereas in adulthood, this inhibitory modulation is absent, coinciding with the emergence of negative affect and stress susceptibility (Przybysz *et al.*, 2017). Early-life stress during infant or juvenile stages can variably increase, leave unchanged, or even downregulate KOR signaling (Karkhanis *et al.*, 2016; Diaz *et al.*, 2018; Lutz *et al.*, 2018), whereas in adults, stress or chronic drug exposure generally enhances KOR function (Knoll & Carlezon, 2010; Karkhanis *et al.*, 2017). Together, these baseline and stress-dependent effects underscore KOR signaling as a context-sensitive,

bidirectional regulator of affect, facilitating emotional resilience during early life and adolescence but contributing to negative mood and maladaptive stress responses in adulthood.

While the MOR system promotes social approach and reinforcement, the DYN/KOR system exerts state- and context-dependent effects on motivation and affect. During adolescence, changes in KOR activity may alter the perceived value of social stimuli, determining whether peer interaction is experienced as rewarding or aversive. Because DYN-mediated and dopaminergic pathways are reciprocally regulated, alterations in KOR function can influence both the drive for social interaction and the capacity to experience it as rewarding. Accordingly, this thesis investigates how endogenous opioid mechanisms, particularly DYN/KOR signaling, contribute to the expression and regulation of social reward across development. Specifically, it examines how developmental changes in the expression of genes coding for the endogenous opioid peptides and receptors relate to social reward, and how pharmacological or genetic inhibition of the DYN/KOR pathway during adolescence alters these behaviors. Through this approach, the work aims to investigate how the maturing brain integrates social and affective cues to generate adaptive motivational states and behavior.

5. Research objective

My thesis investigates how the endogenous opioid system, particularly the DYN/KOR signaling, regulates developmental changes in social reward during adolescence - a period of neural and motivational remodeling when social interaction gains strong affective significance. The aim was to identify the behavioral and molecular mechanisms underlying this process.

The first objective was to establish a behavioral framework for measuring peer-social reward in mice using the conditioned place preference (CPP) paradigm. I validated this approach in adult animals to define baseline levels of social reward and assess potential sex differences in social motivation and reward sensitivity. Next, as adolescence is a period of heightened reward sensitivity and increased motivation for social engagement, I tested whether social reward follows a developmental trajectory distinct from other reward modalities. To address this, I compared CPP responses to social interaction, palatable food, and cocaine across early, mid, and late adolescence. To identify the neurobiological mechanisms underlying these behavioral changes, I examined age-related changes in the endogenous opioid system within the selected corticolimbic regions undergoing substantial adolescent reorganization - the prefrontal cortex, nucleus accumbens, and dorsal striatum. To assess developmental changes, I measured the expression of opioid genes encoding dynorphin (*Pdyn*), enkephalin, and μ , δ, and κ opioid receptors, providing a comprehensive profile of opioid system maturation. Using RNAscope *in situ* hybridization, I further spatially mapped *Pdyn* expression to characterize cell populations undergoing age-dependent reorganization.

Finally, I investigated the functional contribution of DYN/KOR signaling to social and drug reward during adolescence by combining CPP testing with pharmacological KOR blockade (norbinaltorphimine) and genetic deletion of *Pdyn*. These experiments allowed me to determine how DYN/KOR system maturation shapes the affective and motivational value of peer-social interactions.

6. Research included in the doctoral thesis

My doctoral thesis is presented in a hybrid format, combining two original research articles (included in this dissertation, Section **10.** *Scientific articles in their original version*) with additional, unpublished research (Section **6.1** *Additional unpublished research methods and techniques*, ***6.2*** *Additional unpublished research results*). I then synthesize and critically evaluate all findings, emphasizing new, previously unreported results (Section **7.** *Discussion* For context, the main findings of the published work are:

- Adult female mice showed stronger prosocial behavior than males, while both sexes equally expressed social reward in a sibling context and were able to recognize the emotional state of peers. This demonstrated that C57Bl/6 mice show measurable social behaviors and could serve as a model for investigating the neural mechanisms underlying social motivation (Section **10.1** Original *paper 1*)

- Adolescent mice exhibited lower sensitivity to social and palatable food rewards compared to adults, while cocaine reward was stable. Normalized comparisons across all reward types revealed that adolescence is associated with reduced conditioned place preference relative to adults, challenging the idea that reward sensitivity is universally heightened during this period (Section **10.2** *Original paper 2*).

The following sections (**6.1** *Additional unpublished research methods and techniques* and ***6.2*** *Additional unpublished research results*) present unpublished results examining the mechanisms underlying these behaviors. I investigate developmental changes in gene expression within the endogenous opioid system and use pharmacological and genetic manipulations to test the causal role of opioid signaling in peer social reward, linking behavioral changes to their neural basis across adolescence.

6.1 Additional unpublished research methods and techniques

Animals

All animals were maintained on a C57BL/6 genetic background and bred at the Animal Facility of the Maj Institute of Pharmacology, Polish Academy of Sciences (Krakow, Poland). Mice were group-housed (2-6 littermates per cage) in standard ventilated Plexiglas cages (dimensions: 325 × 170 mm), containing nesting material and wooden enrichment blocks. Environmental conditions were strictly controlled: temperature was maintained at 22 ± 2 °C, relative humidity at 40-60%, and animals were kept on a 12:12 h light/dark cycle (lights on at 07:00). Standard laboratory chow (Altromin 1324, Germany) and tap water were provided ad libitum. All procedures were approved by the 2nd Local Bioethics Committee in Krakow (approval numbers: 305/2020, 293/2020, 21/2022, 231/2024) and were carried out in compliance with the European Directive 2010/63/EU and Polish animal welfare regulations (Dz.U. 2015 poz. 266). A separate cohort of animals was used in each experiment described in this chapter. Detailed characteristics of animal age, sex, and body weight at the onset of experimental procedures are provided in **Tables 1-6** (pages 30-32)**.** All experimental procedures were designed and reported in accordance with the ARRIVE guidelines (Percie du Sert *et al.*, 2020).

Pdyn knock-out mouse line

The *Pdyn* knock-out (KO) mouse strain (allele: $Pdyn^{tm1Zim}$) was generously provided by Professor Andreas Zimmer and obtained through the INFRAFRONTIER/EMMA repository (RRID: IMSR_EM:02267) at the EMMA node hosted by the Helmholtz Zentrum München - German Research Center for Environmental Health (INFRAFRONTIER Consortium, 2015). This genetically modified strain carries a targeted disruption of the prodynorphin (*Pdyn*) gene, in which exon 3 and part of exon 4, including the ATG start codon, were replaced by a neomycin resistance cassette. Homozygous *Pdyn*-deficient mice display no overt developmental abnormalities, are viable and fertile, and exhibit normal parental behavior (Zimmer *et al.*, 2001).

Table 1. Age, weight, and number of animals in the qPCR experiment

Sex	Age bin	Animals tested (n)	Subject's weight [g]		Subject's age (days)		Number of outliers in the sample*	
			Mean ± SEM	Range	Mean ± SEM	Range	Brain structure	Gene transcript (n)
Males	E-ado	11	15.8 ± 0.8	7.5	32.2 ± 0.2	2	PFC	*Penk* (1)
							DS	*Pdyn* (1)
							NAc	-
	M-ado	12	18.0 ± 0.4	5.3	37.5 ± 0.2	2	PFC	*Penk* (1), *Pdyn* (1), *Oprk1*(2), *Drd1*(1), *Drd2* (1)
							DS	*Penk* (1), *Pdyn* (1)
							NAc	*Drd1*(1), *Drd2* (2)
	L-ado	12	20.7 ± 0.3	3.5	43.5 ± 0.2	2	PFC	*Penk* (2), *Drd1*(1), *Drd2* (1)
							DS	*Pdyn* (1), *Oprm1*(1)
							NAc	-

*Data points were excluded based on the ROUT outlier test

E-, M-, L-ado - early-, mid-, late-adolescent, **PFC** - prefrontal cortex, **DS** - dorsal striatum, **NAc** - nucleus accumbens, ***Penk*** - preproenkephalin, ***Pdyn*** - prodynorphin, ***Oprk1*** - κ opioid receptor, ***Oprm1*** - μ opioid-receptor, ***Drd1*** - dopamine receptor 1, ***Drd2*** – dopamine receptor 2

Table 2. Age, weight, and number of animals in the RNAscope experiment

Sex	Age bin	Initial animals (n)	Subject's weight [g]		Subject's age (days)		Number of samples included in analysis			
			Mean ± SEM	Range	Mean ± SEM	Range	Brain structure	Animals in probe set[A,B] (n)	Brain sections in probe set[A,B] (n)	Outliers (n)*
Males	E-ado	5	16.2 ± 0.5	6.6	31.8 ± 0.2	2	PFC	2^A, 3^B	4^A, 5^B	-
							DS	2^A, 3^B	7^A, 8^B	-
							NAc	2^A, 3^B	7^A, 8^B	-
	M-ado	5	17.5 ± 0.4	3.4	37.5 ± 0.2	1	PFC	2^A, 3^B	4^A, 4^B	-
							DS	2^A, 2^B	6^A, 7^B	-
							NAc	2^A, 2^B	5^A, 7^B	-
	L-ado	7	19.4 ± 0.5	4.3	43.2 ± 0.1	1	PFC	3^A, 4^B	5^A, 5^B	*Pdyn*_ median (1)
							DS	2^A, 1^B	6^A, 4^B	-
							NAc	2^A, 1^B	6^A, 4^B	-

[A]Scl32a/Sst/Pdyn, [B]Oprd1/Oprk1/Pdyn

*Data points were excluded based on the ROUT outlier test in a selected parameter

E-, M-, L-ado - early-, mid-, late-adolescent, **PFC** - prefrontal cortex, **DS** - dorsal striatum, **NAc** - nucleus accumbens, ***Pdyn*** - prodynorphin

Table 3. Animals used in social CPP with norBNI treatment

Sex	Age bin	Treatment	Animals tested (n)	Subject's weight [g]		Subject's age (days)		Outliers (n)*
				Mean ± SEM	Range	Mean ± SEM	Range	
Males	E-ado	Control	15	14.1 ± 0.7	8.6	31 ± 0.2	2	-
		norBNI	11	13.2 ± 1.0	9.2	31 ± 0.3	3	-
	M-ado	Control	15	17.9 ± 0.4	7.1	37 ± 0.2	3	1
		norBNI	16	18.1 ± 0.4	6.3	37 ± 0.3	3	-
	L-ado	Control	14	18.9 ± 0.2	3.3	43 ± 0.3	4	-
		norBNI	12	18.8 ± 0.4	4.1	43 ± 0.4	4	2

*Data points were excluded based on: Exclusion criterion 1: initial context preference (>70% time in one compartment during pre-test). No data points were excluded based on ROUT outlier test applied to index and distance moved. **E-, M-, L-ado -** early-, mid-, late-adolescent.

Table 4 Animals used in cocaine CPP under norBNI administration

Sex	Age bin	Treatment	Animals tested (n)	Subject's weight [g]		Subject's age (days)		Outliers (n)*
				Mean ± SEM	Range	Mean ± SEM	Range	
Males	E-ado	Control	6	13 ± 0.5	3.1	32 ± 0.2	1	-
		norBNI	7	13 ± 0.6	4.7	31 ± 0.5	3	-
	M-ado	Control	7	18 ± 0.2	1.7	38 ± 0.2	1	-
		norBNI	6	17 ± 0.5	3.2	38 ± 0.2	1	-
	L-ado	Control	9	20 ± 0.3	3.3	44 ± 0.0	0	-
		norBNI	8	20 ± 0.3	2.2	44 ± 0.0	0	-

*No data points were excluded based on: Exclusion criterion 1: initial context preference (>70% time in one compartment during pre-test) or ROUT outlier test applied to index and distance moved. **E-, M-, L-ado -** early-, mid-, late-adolescent.

Table 5. Animals used in phenotype characterization of adult *Pdyn* KO mice

Test	Sex	Genotype	Animals tested (n)	Subject's weight [g]		Subject's age (weeks)		Outliers (n)*
				Mean ± SEM	Range	Mean ± SEM	Range	
Open field	Female	Control	10	5.8 ± 9.0	4	12.9 ± 0.6	4	-
		Pdyn KO	9	21.0 ± 0.6	6	14.1 ± 0.7	7	-
	Male	Control	9	26.8 ± 1.0	9	15.3 ± 0.3	2	-
		Pdyn KO	7	27.6 ± 1.2	8	14.9 ± 0.9	7	-
Light-dark box	Female	Control	10	20.1 ± 1.1	11	14.5 ± 1.2	9	-
		Pdyn KO	12	20.9 ± 0.7	8	15.1 ± 1.0	9	-
	Male	Control	11	23.4 ± 0.7	8	12.5 ± 0.7	5	-
		Pdyn KO	9	25.6 ± 0.7	8	13.7 ± 0.8	7	-
Saccharin preference	Female	Control	6	NA[1]		13.2 ± 1.5	9	-
		Pdyn KO	17			14.8 ± 0.8	9	-
	Male	Control	11	NA[1]		12.7 ± 0.7	5	-
		Pdyn KO	9			14.0 ± 0.8	7	-

*No data points were excluded based on the ROUT outlier test applied to: OF = distance moved. LDB = total time in light box
[1]Data lost

Table 6. Animals used in social CPP with adolescent *Pdyn* KO mice

Sex	Age bin	Treatment	Animals tested (n)	Subject's weight [g]		Subject's age (days)		Outliers (n)[1,2]
				Mean ± SEM	Range	Mean ± SEM	Range	
Males	E-ado	Control	12	13 ± 0.7	8.4	28 ± 0.6	6	1^{1}, 2^{3}
		Pdyn KO	17	12 ± 0.3	5.2	26 ± 0.2	2	1^{3}, 2^{5}
	M-ado	Control	9	14 ± 1.3	10.3	31 ± 0.3	2	2^{2}
		Pdyn KO	11	17 ± 0.3	3.5	32 ± 0.3	2	1^{1}, 3^{2}
	L-ado	Control	9	18 ± 0.7	5.8	37 ± 0.2	2	1^{3}
		Pdyn KO	14	17 ± 0.4	5.1	38 ± 0.1	1	1^{2}, 2^{2}

Data points were excluded based on: [1]Exlusion criterion 1: initial context preference (>70% time in one compartment during pre-test), [2]Exlusion criterion 2: social-paired context matched the individual's most-preferred compartment. No data points were excluded based on ROUT outlier test applied to the index and distance moved. **E-, M-, L-ado -** early-, mid-, late-adolescent.

Real-time quantitative PCR (qPCR)

Tissue collection and preparation

Mice were euthanized by cervical dislocation followed by decapitation. Whole brains were rapidly extracted and immediately placed in 2 ml of RNAlater (Thermo Fisher Scientific) for 1 hour at room temperature (RT), then stored at 4°C for 48 hours. Brains were sectioned coronally at 120 µm thickness in RNAlater on vibratome (Leica VT1299). Dissections of the mPFC (comprising the cingulate cortex, prelimbic cortex, infralimbic cortex, and dorsal peduncular cortex), DS, and NAc were performed under a stereomicroscope (Zeiss StemiDV4) using needles. Target brain regions were identified based on stereotaxic coordinates from the Franklin and Paxinos Mouse Brain Atlas (Paxinos & Franklin, 2001) corresponding approximately to Bregma coordinates 1.94 to 0.62 mm. Microdissected tissue samples were transferred to 200 µl RNAlater, which was subsequently removed by aspiration, and samples were stored at −20°C until further processing.

RNA isolation

Tissue samples were homogenized in 1 ml TRIzol reagent (Invitrogen) using 2 ml round-bottom centrifuge tubes with steel beads in a TissueLyser II (Qiagen) at 30 Hz for 3 minutes. To ensure uniform tissue disruption and compensate for possible differences in shaking intensity or adapter position, the tubes with tissue samples were swapped within the adapter set and subjected to a second 3-minute homogenization. Total RNA was isolated via the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987), followed by additional purification using an RNeasy Mini Kit (#74904, Qiagen) according to the manufacturer's instructions. RNA concentration was measured with a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific).

Reverse transcription

Complementary DNA (cDNA) synthesis was performed using the Omniscript reverse transcription Kit (#205113, Qiagen) with Oligo(dT)$_{12–18}$ primers (#18418012, Invitrogen), following the manufacturer's protocol. Synthesized cDNA was stored at −20°C until use.

qPCR

Quantitative gene expression analysis was conducted using qPCR with Universal PCR Master Mix (#4304437, Thermo Fisher Scientific) and TaqMan Gene Expression Assays targeting *Penk* (preproenkephalin gene, Mm01212875_m1), *Pdyn* (prodynorphin gene, Mm00457573_m1), *Oprk1* (κ opioid receptor gene, Mm01230885_m1), *Oprm1* (μ opioid receptor gene, Mm01188089_m1), *Oprd1* (δ opioid receptor gene, Mm01180757_m1), *Drd1* (dopamine receptor 1 gene, Mm01353211_m1), *Drd2* (dopamine receptor 2 gene, Mm00438545_m1), and the reference gene *Hprt1* (hypoxanthine phosphoribosyltransferase 1 gene, Mm03024075_m1). Reactions were run on a C1000 thermal cycler with a CFX96 optical module (BioRad). Relative gene expression was calculated using the comparative Ct (cycle threshold) method, with normalization to *Hprt1* as an endogenous control. Expression levels were calculated using the formula: $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{target\ gene} - Ct_{Hprt}$.

In situ **hybridization RNAscope**

Tissue collection and preparation of fresh-frozen sections

Mice were euthanized via cervical dislocation, and brains were extracted on ice. Following a saline rinse to remove blood, brains were blotted dry, placed cerebellum-down on aluminum foil, and snap-frozen on dry ice. Once frozen (~1 minute), brains were embedded in chilled Peel-A-Way molds (#18646A-1, Polysciences) filled with Optimal Cutting Temperature compound (OCT; #KMA-0100-00A, CellPath) and stored at −80°C. For sectioning, embedded blocks and tools (slides, brushes, tweezers) were equilibrated to cryostat temperature (−20 to −22°C; Leica CM3050 S) for at least 30 minutes. Coronal sections (10 μm thick) were cut and mounted onto SuperFrost Plus slides (Thermo Fisher Scientific) using a fine brush. Adhesion was achieved by briefly pressing the back of the slide with a finger. Slides were air-dried in the cryostat chamber for ~1 hour and stored at −80°C. Sections containing regions of interest (ROIs, i.e mPFC, DS, NAc) were identified based on the stereotaxic coordinates from the Franklin and Paxinos Mouse Brain Atlas (Paxinos & Franklin, 2001) spanning approximately bregma 1.54 to − 1.10 mm.

Fixation and dehydration

Slides were removed from −80°C and immediately fixed in ice-cold 4% (w/v) paraformaldehyde (pH 7.4) for 20 minutes at 4°C. Tissue was rinsed twice in 1× PBS (Merck Millipore), followed by graded ethanol dehydration (50%, 70%, 100%, 100%, 5 min each, RT). After drying for 5 minutes on absorbent paper, a hydrophobic barrier (~1.9 × 1.9 cm) was drawn around the tissue sections using an Immedge hydrophobic barrier pen (#310018, Vector Laboratories). Slides were air-dried for 1 minute to allow barrier polymerization.

Tissue pretreatment and probe hybridization

RNAscope Multiplex Fluorescent v2 Assay (#323100, ACD Bio) was performed according to the manufacturer's protocol. Briefly, sections were treated with hydrogen peroxide for 10 minutes at RT to quench endogenous peroxidase activity, followed by Protease IV digestion (30 minutes, RT). Target-specific probes were applied and incubated for 2 hours at 40°C in a humidified chamber. Probes were pre-warmed to 40°C for 10 minutes and equilibrated to RT for 10 minutes prior to use.

Probes used:

- Mm-Pdyn (prodynorphin; #318771-C1)
- Mm-Oprk1 (κ opioid receptor; #316111-C2)
- Mm-Sst (somatostatin; #404631-C2)
- Mm-Slc32a1 (vesicular GABA transporter; #319191-C3)
- Mm-Oprd1 (δ opioid receptor; #427371-C3)
- Positive control: *Polr2a* (polymerase 2 subunit A; C1), *Ppib* (peptidylprolyl isomerase B; C2), *Ubc* (polyubiquitin; C3) (#320861)
- Negative control: bacterial gene *dapB* (4-hydroxy-tetrahydrodipicolinate reductase; C1–C3) (#320871)

Probe sets:

- Set A: *Pdyn* (C1), *Sst* (C2), *Slc32a1* (C3)
- Set B: *Pdyn* (C1), *Oprk1* (C2), *Oprd1* (C3)

Signal amplification was performed sequentially: Amp 1-FL (30 min, 40°C), Amp 2-FL (15 min, 40°C), and Amp 3-FL (30 min, 40°C), followed by incubation with TSA Vivid fluorophores (#323270, ACD).

Fluorophores were reconstituted in DMSO (100 µL) and diluted 1:1500 in working buffer prior to application. The fluorophore-to-channel configuration was:

- C1: TSA 650 (Red)
- C2: TSA 570 (Yellow)
- C3: TSA 520 (Green)

To minimize photobleaching, all steps from fluorophore application onward were performed under low-light conditions with aluminum foil shielding during wash steps. Signal development was performed using sequential HRP-based reactions.

Counterstaining and mounting

Following signal development, sections were counterstained with DAPI (30 seconds, RT), then mounted with ProLong Gold Antifade Mountant (#P36930, Thermo Fisher Scientific) and coverslipped. Coverslip edges were sealed with fast-drying nail polish to prevent leakage. Slides were stored at 4°C in the dark and imaged within 8-48 hours.

Imaging

Fluorescent imaging was performed using a Leica THUNDER 3D Live Cell fluorescence microscope (DMI8, Leica Microsystems) with a 20× dry UV objective (HC PL APO CS2, NA: 0.75). Z-stacks (8-12 µm depth, 15-20 slices). Imaging settings per channel were:

- Channel 1 (DAPI, blue): 55 ms
- Channel 2 (TSA 520, green): 120 ms
- Channel 3 (TSA 570, yellow): 80 ms
- Channel 4 (TSA 650, red): 80 ms

Quantification

Z-stacks were processed into maximum intensity projections. Channels were merged using ImageJ in the following order: DAPI (blue), *Slc32a1/Oprd1* (green), *Sst/Oprk1* (yellow), *Pdyn* (red). Quantification was conducted in QuPath v0.5.1 (Bankhead *et al.*, 2017), with methods adapted from Secci et al. (Secci *et al.*, 2023). First, ROIs (mPFC, DS, NAc) were delineated manually using atlas-based landmarks. Nuclei were detected on the DAPI channel using the following parameters:

- Background radius: 10 µm
- Median filter radius: 2.0 µm
- Sigma: 2.0 µm
- Max area: 300 µm²
- Intensity threshold: 2000

Detected nuclei were used to define cell boundaries. RNAscope puncta were quantified using QuPath's subcellular detection tool with the following settings:

- Expected spot size: 0.5 µm²
- Min/Max spot size: 0.5/15 µm²
- Smooth before detection: Yes
- Split by intensity and shape: Yes
- Include clusters: Yes

Detection thresholds were empirically optimized to ensure accurate spot identification per channel:

- Channel 2: 1200–1800
- Channel 3: 15,000–30,000
- Channel 4: 800–5000

Data were exported and analyzed using R Statistical Software (v4.5.0, R Core Team, 2025) The script used for this study can be found in the Zenodo repository (10.5281/zenodo.17582213). Only cells with >5 puncta were included in downstream analyses. The following metrics were calculated:

1. Median number of spots per cell
2. H-score**,** weighted staining intensity score calculated as:

   H-score = ∑(Bin number × % cells in bin), where binning was as follows:

   - Bin 0: No staining/<1 dot per 10 cells
   - Bin 1: 1–3 dots/cell
   - Bin 2: 4–9 dots/cell
   - Bin 3: 10–15 dots/cell or <10% dots in clusters
   - Bin 4: >15 dots/cell or >10% dots in clusters

     (Score range: 0–400)
3. Colocalization**:** Total number of cells showing >5 dots for two or more probes.

Pdyn KO validation by RNAscope

To confirm *Pdyn* deletion in knockout animals, RNAscope *in situ* hybridization was performed for qualitative visualization of expression patterns; no quantitative analysis was conducted. Coronal sections encompassing the striatum were collected at approximately +1.18 mm relative to Bregma. The following probes and fluorophores were used:

- Mm-Pdyn (C1, #318771), detected with Atto 550 (orange fluorescence, #320850),
- Mm-Penk (C2, #318761), detected with Atto 647 (red fluorescence, #320850), and
- Mm-Oprm1 (C3, #315841), detected with Alexa Fluor 488 (green fluorescence, # 320850)

Behavioral methods

General information

All behavioral experiments were conducted during the light phase under dim lighting conditions (5–10 lux) with supplemental infrared illumination. Mice were handled daily for 2-3 minutes over a period of 3-5 days prior to testing. Tail markings were refreshed every 3 days for individual identification. On test days, animals were acclimated to the behavioral room for at least 30 minutes prior to the start of each session. Locomotor activity and spatial tracking were recorded via a digital camera (acA1300–60 gm, Basler, Germany) and analyzed using EthoVision XT 15 software (Noldus, The Netherlands) with two exceptions. Social interaction in the open field was video recorded and manually scored using BORIS software (Friard & Gamba, 2016), while saccharin preference was quantified by measuring fluid consumption.

Conditioned place preference (CPP)

Animals were housed with same-sex littermates on aspen bedding (ABEDD, Latvia or Tapvei GLP) prior to testing. CPP consisted of three phases: pre-test, conditioning, and post-test. The two-compartment CPP apparatus featured distinct contextual cues in each chamber: one contained beech shavings (P.P.H. "WO-JAR", Poland) and cuboid wooden blocks (Zoolab, Poland), while the other had pelleted cellulose bedding (#L0107, 1/8", Scott Pharma Solutions) and cube-shaped blocks (Zoolab, Poland). During the 30-minute pre-test, mice were allowed to freely explore both compartments to assess baseline context preference. A biased design was used: the less-preferred context was paired with the

rewarding stimulus during conditioning. Conditioning was conducted in new home cages containing the assigned contextual cues. To prevent visual exposure to littermates (due to cage transparency), opaque cardboard dividers were placed between cages. The post-test occurred the day after the final conditioning session under identical conditions to the pre-test. CPP index was calculated as the change in time spent in the reward-paired context (post-test minus pre-test).

Social CPP

Social CPP was based on a modified version of the protocol developed by Panksepp and Lahvis (Panksepp & Lahvis, 2007) with adaptations by Dölen, Nardou and colleagues (Dölen *et al.*, 2013; Nardou *et al.*, 2019) and modified as described in our previous studies (Harda *et al.*, 2022; Misiołek *et al.*, 2023; Harda *et al.*, 2025a, 2025b). Modifications introduced in this study included: 1) a biased contextual design, and 2) a reduction in the number of conditioning sessions. Due to the group-based design, in which littermates served as both subjects and social stimuli, individual preference could not be used to assign context. Instead, initial preference indices were averaged across cage-mates, and the less-preferred context (based on group means) was paired with the social condition. Conditioning consisted of alternating 24-hour sessions of social housing (with littermates) and isolation over four consecutive days. Each mouse received both conditions, starting with social exposure 2 hours after the pre-test (to minimize potential interference from pre-test exposure). Mice meeting exclusion criteria completed the full procedure but were omitted from final analyses (see **Table 3** and **Table 6**). Two exclusion criteria were applied: animals were excluded if they showed a strong initial context preference (>70% time in one compartment during pre-test) or if their social-paired context matched the individual's preferred compartment (animal's initial preference opposite to cage-mates; used as stimulus, not as subject).

Cocaine CPP

Cocaine CPP followed the same design as the social CPP, using a biased procedure with alternating cocaine and saline conditioning sessions, where the individual-based less-preferred context was paired with cocaine hydrochloride (10 mg/kg, i.p., 5 µL/g, dissolved in 0.9% saline; Toronto Research Chemicals), and the more-preferred context was paired with saline (0.9%, 5 µL/g; Polpharma, Poland). Conditioning was conducted over three consecutive days, with two daily sessions (40 minutes each) separated by a 4-hour inter-trial interval. Morning sessions involved saline injections, and afternoon sessions involved cocaine. As in social CPP, all mice completed the procedure, but not all animals were included in the final analysis (see **Table 4**). One exclusion criterion was applied: mice that spent more than 70% time in one compartment during pre-test were excluded.

CPP with norBNI administration

To test the role of KOR signaling, a subset of mice received the selective KOR antagonist norbinaltorphimine dihydrochloride (norBNI; TOCRIS, Cat. No. 0347, UK), administered intraperitoneally at 10 mg/kg (5 µL/g) dissolved in 0.9% saline. Injections were performed 1 hour after the pre-test in both social and cocaine CPP paradigms.

To prevent control mice from interacting with norBNI-treated cage-mates, animals were subdivided into smaller groups after pre-test, and each sub-cage was randomly assigned to receive norBNI or vehicle (saline). NorBNI is a long-acting KOR antagonist (Kishioka *et al.*, 2013, up to 4 weeks), but exhibits transient off-target MOR and DOR inhibition within the first 2 hours post-injection (Endoh *et al.*, 1992; Horan *et al.*, 1992). To minimize potential MOR involvement, the first social CPP conditioning session began 2 hours after injection. In the case of cocaine, conditioning started the following day.

Open field test

Exploratory behavior was assessed in a white square-shaped arena (40 × 40 × 40 cm) filled with 0.5 cm of aspen bedding and dimly illuminated (6-10 lux). Mice were individually placed in the center and allowed to explore freely for 30 minutes. A central zone (defined as 5 cm from each wall) was used to evaluate center-avoidance. Recorded parameters included: total distance traveled (m) and time spent in the center zone (% of total time).

Social interaction in an open field

Social behavior was evaluated immediately after the open field test, which also served as a habituation phase. A novel, age- and sex-matched conspecific was introduced, and mice were allowed to interact freely for 10 minutes. Social interaction was defined as the total time spent in direct behaviors initiated by the test mouse, including sniffing (anogenital, body, or head), grooming, chasing, and mounting.

Light-dark box test

Anxiety-like behavior was assessed using a standard light–dark box consisting of two compartments (i.e, boxes, 18 × 16 × 20 cm): one brightly lit (400 lux), and one dim (50 lux), connected by a small opening. Mice were placed in the dark compartment and allowed to explore for 5 minutes. Measured parameters included: latency to enter the light box (s) and total time spent in the light compartment %.

Saccharin preference test

To evaluate anhedonia-like behavior, we used a two-bottle saccharin preference test. Mice were single-housed for 24 hours in standard cages (26 × 20 × 13.5 cm) containing two 25 ml bottles of tap water. On the test day, one bottle was replaced with 0.1% (w/v) saccharin solution. Bottle positions were randomized. Food was available *ad libitum*. After 24 hours, fluid levels were manually recorded. Saccharin preference was calculated as: saccharin preference % = (saccharin intake (ml) / total solution intake (ml)) × 100.

Data analysis

All statistical analyses were performed using GraphPad Prism 10.4.2 (GraphPad Software), unless otherwise stated. Outliers were identified using the ROUT test and are reported in **Tables 1-6** (pages 30-32) together with the numbers of animals excluded based on pre-test criteria. Gene expression data were analyzed using the Kruskal-Wallis test followed post hoc by Dunn's test. Behavioral data were analyzed using ANOVA with Tukey's HSD post hoc. Statistical tests and sample sizes are reported in the main text and/or figure legends.

6.2 Additional unpublished research results

Maturation of opioid gene expression during adolescence

First, I examined the expression of opioid-related genes across three corticolimbic regions undergoing pronounced structural and functional remodeling during adolescence: the mPFC, the DS, and the NAc in C57BL/6 male mice. In line with our previous experiments, three adolescent stages were examined: early-adolescence (~postnatal day 32; puberty onset), mid-adolescence (~P38; peripubertal), and late-adolescence (~P43; sexual maturity). To quantify transcript abundance corresponding to expression within entire target structures, analyses were performed on tissue homogenates prepared from brain sections. Discrete samples of each region were collected using needle punches under a binocular microscope and subsequently pooled for molecular analysis (**Figure 2A**).

Developmental changes in expression of opioid-related genes

qPCR revealed regionally distinct and temporally coordinated developmental trajectories in opioid gene expression. In both the mPFC and the DS, relative *Pdyn* transcript levels declined significantly from early to mid-adolescence (mPFC: **Figure 2B**, $H = 7.34$, $p = .0325$; DS: **Figure 2G**, $H = 13.8$, $p = .001$), whereas relative *Penk* (preproenkephalin) expression remained stable (mPFC: **Figure 2C**; DS: **Figure 2H**, $p > 0.05$). In contrast, mRNA levels of the three canonical opioid receptors genes: *Oprk1, Oprm1*, and *Oprd1* did not vary significantly across ages (mPFC: **Figure 2D-F**; DS: **Figure 2I-K,** $p > 0.05$). This selective reduction in *Pdyn* transcript suggests a temporal reorganization of inhibitory opioid signaling in cortical and dorsal striatal networks, potentially adjusting neuromodulatory tone without altering receptor availability.

In the NAc, the relative expression of both *Pdyn* and *Penk* transcripts remained unchanged with age (**Figure 2L-M,** $p > 0.05$). However, *Oprd1* expression increased progressively from early- to late-adolescence (**Figure 2P**, $H = 9.8$, $p = .008$), whereas *Oprk1* and *Oprm1* expression remained stable (**Figure 2N-O,** $p > 0.05$).

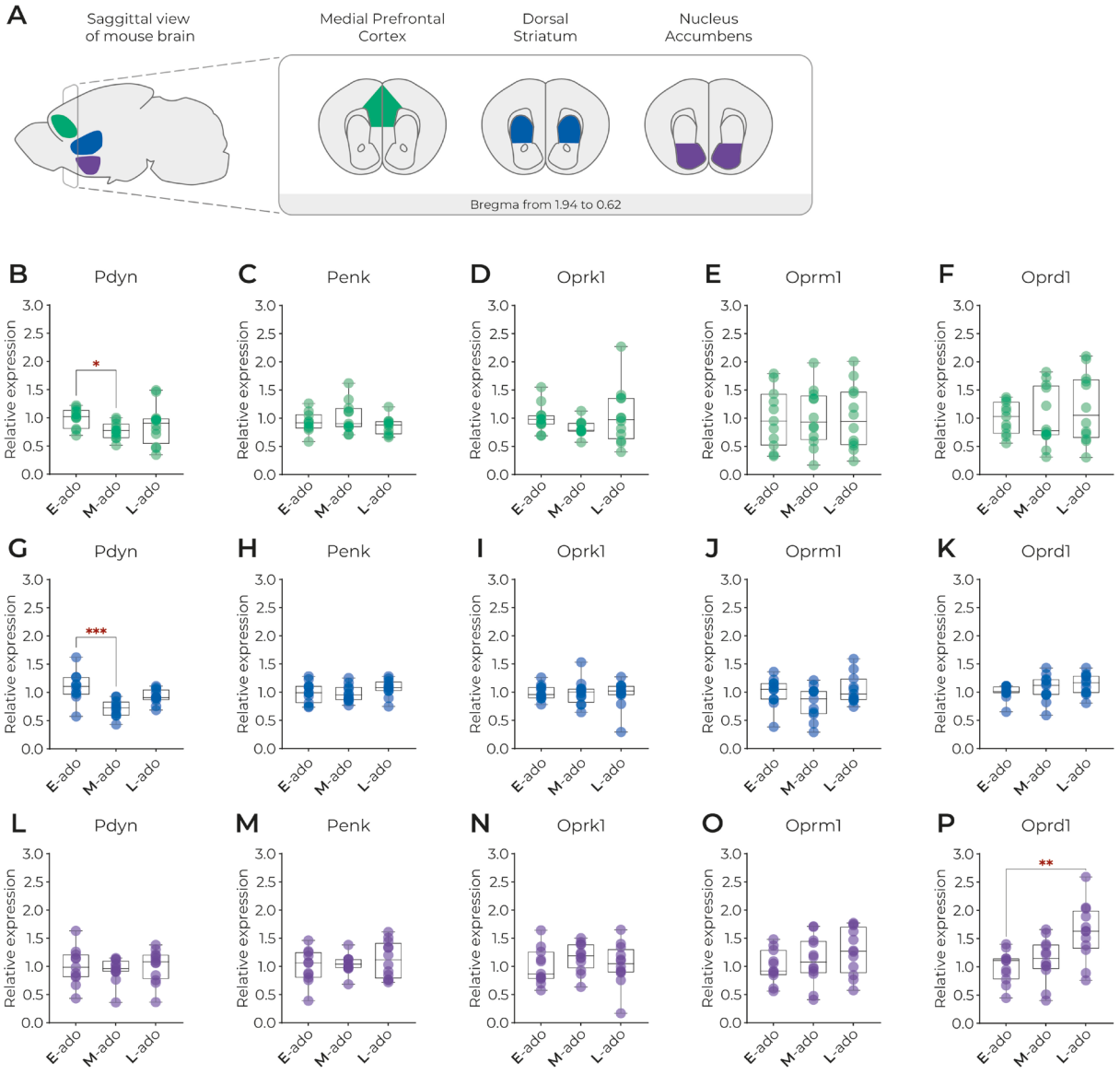

Figure 2. Developmental changes in expression of opioid-related genes

(A) Schematic representation of the mouse brain (sagittal view) with color-coded regions: medial prefrontal cortex (mPFC, green), dorsal striatum (DS, blue), and nucleus accumbens (NAc, purple). Tissue samples were obtained using needle dissection from brain sections spanning bregma coordinates 1.94 to –0.62 mm.

(B–F) Relative expression of *Pdyn*, *Penk*, *Oprk1*, *Oprm1*, and *Oprd1* in the mPFC;
(G–K) in the DS; and
(L–P) in the NAc.

Boxplots represent interquartile ranges with median values, and whiskers indicate the range. Each circle represents an individual animal. Statistical analyses were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test, with significance indicated as $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Together, these data reveal that the adolescent opioid landscape undergoes region-specific remodeling, marked by a coordinated reduction in cortical and dorsal striatal *Pdyn* expression and a concurrent upregulation of *Oprd1* in the NAc.

Next, I quantified the relative abundance of dopamine 1 (*Drd1*) and dopamine 2 (*Drd2*) receptor transcripts. The expression of *Drd2* in the DS and *Drd1* in the NAc were significantly higher in late- compared to early-adolescent mice (DS: **Figure 3D**, H = 7.8, p = .012; NAc: **Figure 3E**, H = 10.4, p = .005). No other significant changes in relative expression of dopamine receptor transcripts were observed across regions (**Figure 3A-B, C, F,** p > 0.05). The observed trends align with previous reports showing a transient peak in dopamine receptor expression during adolescence (Reynolds & Flores, 2021; Avramescu *et al.*, 2024). Collectively, these data suggest that dopamine and opioid systems mature in opposite but coordinated ways during this critical period of brain development, shaping the transition from adolescent to adult reward sensitivity.

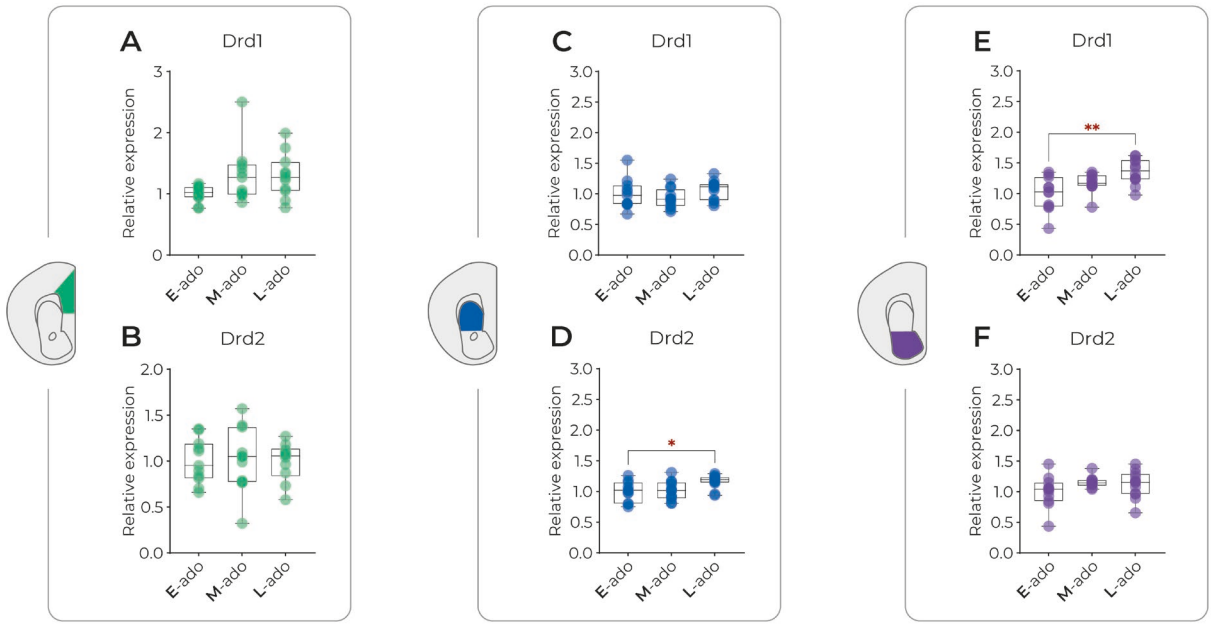

Figure 3. Developmental changes in expression of dopamine receptor genes

(A–B) Relative expression of dopamine receptor 1 (*Drd1*) and dopamine receptor 2 (*Drd2*) genes in the mPFC;
(C–D) in the DS; and
(E–F) in the NAc.

Boxplots represent interquartile ranges with median values, and whiskers indicate the range. Each circle represents an individual animal. Statistical analyses were performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Significance levels are indicated as p ≤ 0.05 (*) and p ≤ 0.01 (**).

Developmental changes in *Pdyn*-positive neurons

Next, to identify the neuronal populations of *Pdyn*-expressing neurons underlying these transcriptional shifts, I complemented the qPCR results with spatially and single-cell resolved analyses, RNAscope *in situ* hybridization.

Optimization of the RNAscope protocol was confirmed using both positive and negative control probes (**Figure 4A&B**). Multiple sections (2-3 per animal) from 12 animals per age bin were prepared, however tissue quality and variability in sectioning reduced the final number of usable sections (~4-8 per brain structure; n = 1-4 animals per age, as reported in **Table 2**). Imaging prioritized the detection of all transcript spots, which sometimes resulted in signal clustering in high-expression regions. For the positive controls, the *Ubc* (polyubiquitin C) transcript - highly expressed in most cell types - was abundantly detected across sections (**Figure 4A**). The *Pol2a* (polymerase II subunit A, low-expression control) and *Ppib* (peptidylprolyl isomerase B, medium-expression control) transcripts were also detected, although signal intensity was lower, consistent with their reported expression levels in the brain, *Ppib* being relatively low and *Polr2a* lowest (**Figure 4A**). In contrast, the *dapB* probe (bacterial 4-hydroxy-tetrahydrodipicolinate reductase; non-mammalian negative control) produced no detectable signal, confirming the absence of nonspecific hybridization (**Figure 4B**). These results verified that RNA integrity and hybridization quality were sufficient for subsequent experimental analyses.

Two experimental probe sets were employed: set1, *Pdyn*, *Sst* (somatostatin), and *Slc32a1* (vesicular GABA transporter), and set2 targeting *Pdyn*, *Oprd1*, and *Oprk1*, each with DAPI nuclear counterstaining (**Figure 4C&D**, respectively). Median cell counts were robust across all regions (mPFC: 7980; DS: 7758; NAc: 6630), with *Pdyn* signal (defined as >5 spots per *Pdyn*-positive cell) detected in 22%, 43%, and 69% of all cells in the mPFC, the DS, and the NAc, respectively.

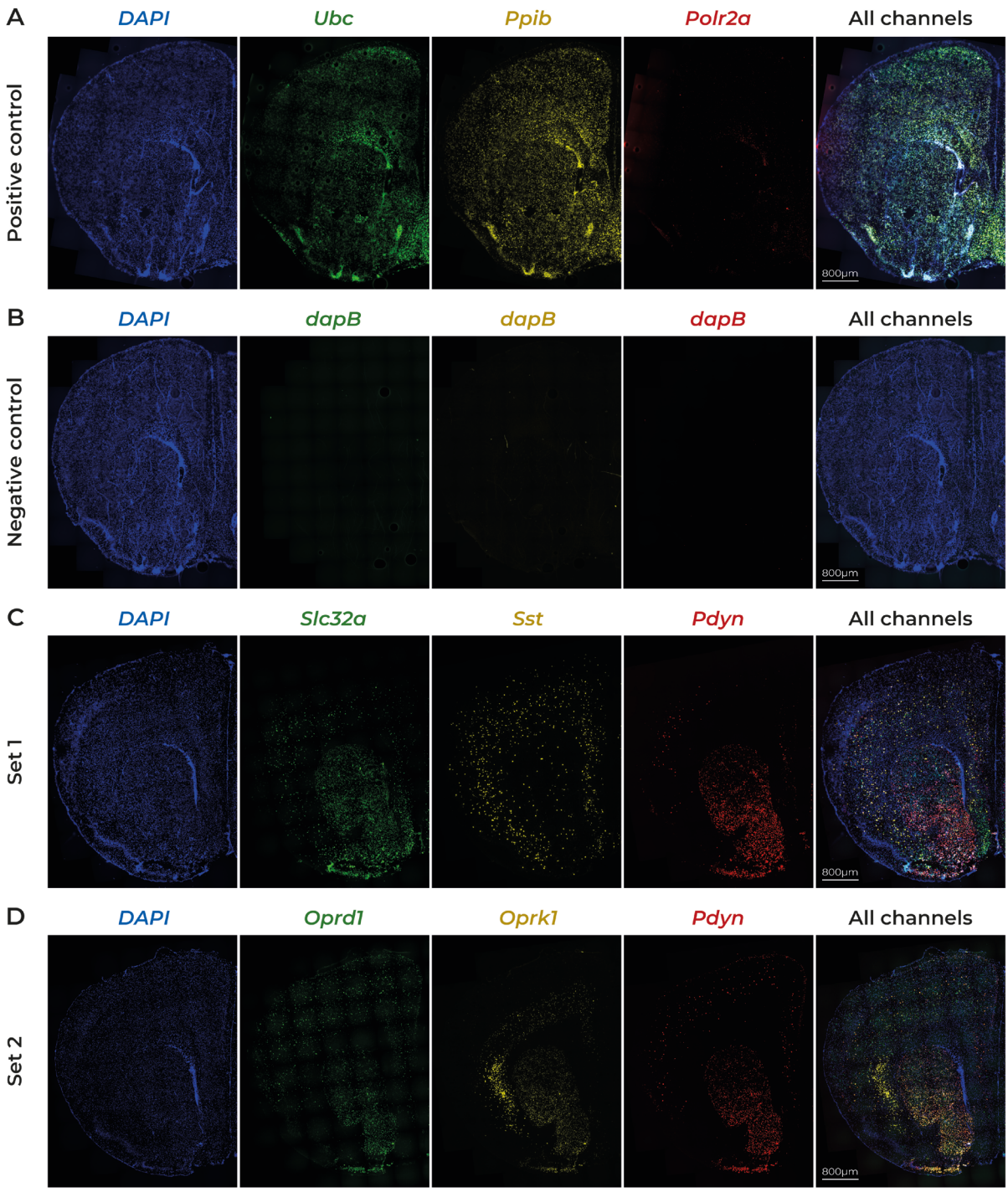

Figure 4. Overview of RNAscope results, including control and experimental probe sets

Exemplary images of each channel (C1-C4) and merged images are shown for the following probe sets:

(A) Positive control: *DAPI*, *Ubc*, *Ppib*, *Polr2a*,
(B) Negative control: *DAPI, dapB,*
(C) Experimental Set1 (cortex-adapted): *DAPI, Slc32a, Sst, Pdyn,*
(D) Experimental Set2 (striatum-adapted): *DAPI, Oprd1, Oprk1, Pdyn.*

Fluorophores used for each channel are: C1: DAPI (blue), C2: TSA 520 (green), C3: TSA 570 (yellow), C4: TSA 650 (red). Scale bars are shown in each image.

Medial prefrontal cortex

Consistent with prior literature, *Pdyn*-expressing neurons were localized to cortical layers II/III and V/VI (Yarur *et al.*, 2023; Wang *et al.*, 2024). The expression of *Pdyn* (**Figure 5B-C,** H-score, and median spots per cell, both $p > 0.05$) did not change across adolescence, in contrast to qPCR results showing a decrease with age. According to previous reports, in mPFC *Pdyn* transcript is predominantly expressed in excitatory neurons (~80%), with a smaller fraction in inhibitory interneurons (~20%). We examined inhibitory populations to assess whether shifts in inhibitory control might underlie developmental changes in cortical excitability or reward-related signaling. Interneurons were detected with probes for *Slc32a1* (GABAergic marker) and *Sst* mRNA, given prior evidence that *Pdyn* is preferentially co-expressed in somatostatin-positive interneurons (**Figure 5D**). The proportion of $Pdyn^{Sst}$ interneurons increased from early to mid-adolescence (**Figure 5F**, $H = 8$, $p = .005$) while $Pdyn^{Slc32a1}$ and $Pdyn^{Slc32a1Sst}$ populations remained stable (**Figure 5G-H,** $p > 0.05$, median 14.5% and 6.8% of all *Pdyn*-positive cells, respectively). Moreover, total expression of each *Sst* (H-score, and median spots per cell, both $p > 0.05$) or *Pdyn* did not change significantly. Cumulatively, these findings indicate a selective developmental expansion of somatostatin-positive inhibitory *Pdyn* neurons, potentially influencing local excitatory-inhibitory balance without substantially altering the overall inhibitory population.

Receptor co-expression analyses (**Figure 5E**) revealed an increase in the percentage of $Pdyn^{Oprk1}$ neurons during mid-adolescence (**Figure 5I,** $H = 8.6$, $p = .004$), while *Pdyn* and *Oprd1* co-expression decreased (**Figure 5J**, $H = 7.7$, $p = .001$); the double-positive population remained stable (**Figure 5K**, $p > 0.05$, median 6.8% of all *Pdyn*-positive cells). The total expression of *Oprk1* and *Oprd1* (H-score, and median spots per cell, both $p > 0.05$) did not change across adolescence. Together, these data suggest that KOR signaling is selectively strengthened in mPFC dynorphin neurons during adolescence, shaping cortical excitability in a temporally specific manner.

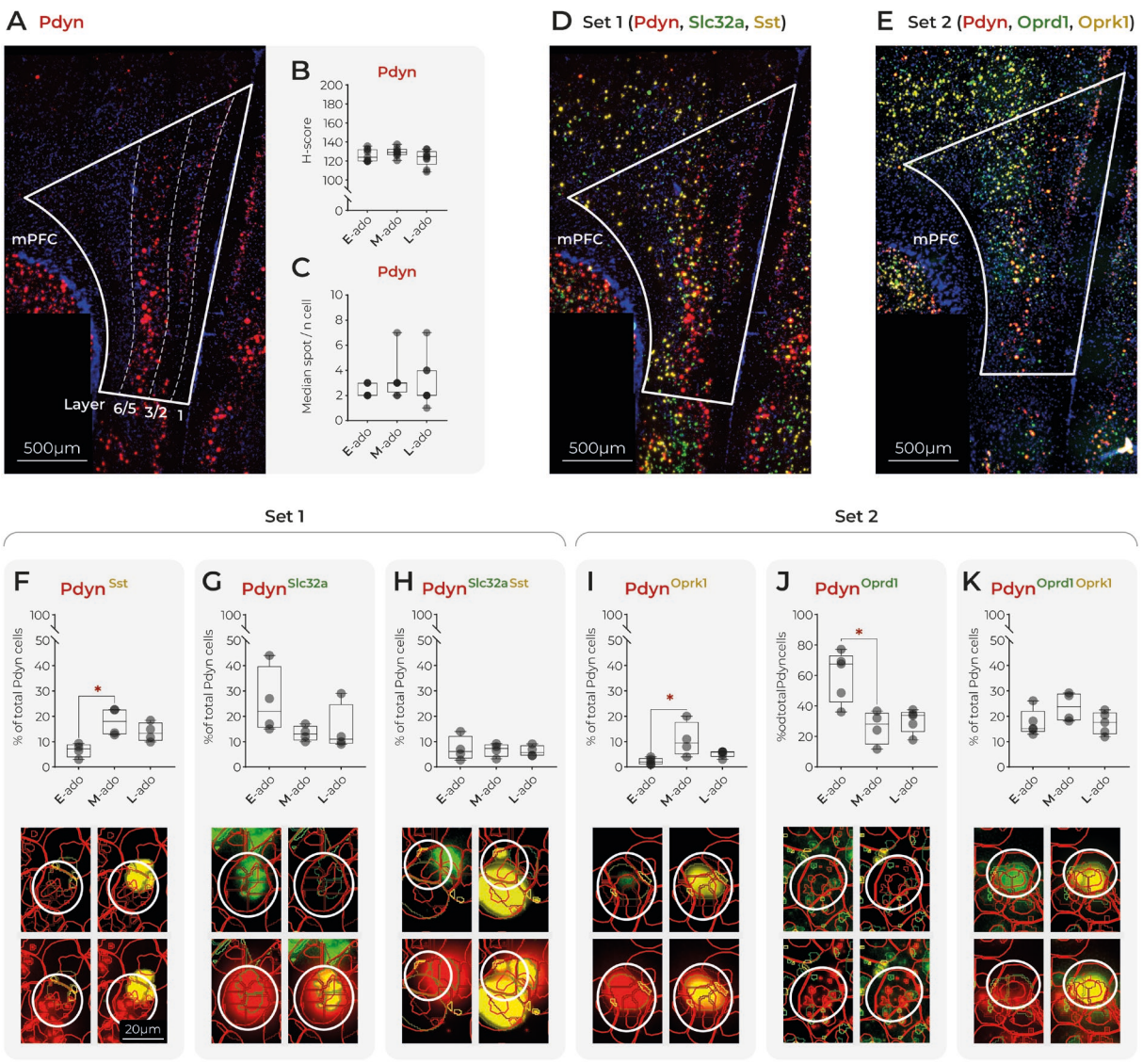

Figure 5. Developmental changes in *Pdyn*⁺ populations in the mPFC

(A) Representative image of *Pdyn* mRNA (red) expression in the mPFC.
(B) *Pdyn* mRNA expression quantified using the H-score.
(C) Median number of *Pdyn* mRNA spots per Pdyn⁺ cell.
(D) Representative images showing mPFC *Pdyn* (red), *Slc32a1* (green), and *Sst* (yellow) mRNA expression.
(E) Representative images showing mPFC *Pdyn* (red), *Oprd1* (green), and *Oprk1* (yellow) mRNA expression.
(F–H) Changes in the fraction of $Pdyn^{Sst^+}$, $Pdyn^{Slc32a^+}$, and $Pdyn^{SstSlc32a^+}$ neurons, respectively.
(I-K) Changes in the fraction of $Pdyn^{Oprk1^+}$, $Pdyn^{Oprd1^+}$ and $Pdyn^{Oprd1Oprk1^+}$ neurons, respectively.

Boxplots represent interquartile ranges with median values, and whiskers indicate the range. Each circle represents an individual animal. Statistical analyses were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test, with significance indicated as $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

For panels **(F–K)**, each graph is accompanied by an example cell indicated with a white circle. Cell nuclei, perimeters, and RNAscope signals were detected and annotated using QuPath software. Each set of four images shows the three individual channels and a merged image. Scale bars are shown in each image.

Dorsal striatum

In the DS, *Pdyn* gene expression showed a patchy, striosome-like distribution (**Figure 6A)**. *Pdyn* transcript is predominantly expressed in dopamine D1 receptor-expressing GABAergic medium spiny neurons (Gerfen & Scott Young, 1988; Gerfen & Surmeier, 2011), and consistent with this, *Sl32a1*, the GABAergic neuron marker, was detected in 86% of Pdyn-expressing cells (**Figure 6F**, **Figure 6D** for overview). The total *Pdyn* levels did not change across ages (**Figure 6B,** H-score, $p > 0.05$, **Figure 6C,** median spots per cell, $p > 0.05$). The population of $Pdyn^{Oprk1}$ neurons significantly increased (**Figure 6G**, $H = 5.7$, $p = .05$, **Figure 6E** for overview), whereas the population of $Pdyn^{Oprd1}$ neurons decreased (**Figure 6H**, $H = 10.4$, $p = .001$); the fraction of double-positive cells remained stable (**Figure 6I**, $p > 0.05$). The overall expression levels of *Oprk1* and *Oprd1*, as assessed by H-score and median spots per cell, did not exhibit significant changes across adolescence (**Figure 6J-K,** both $p > 0.05$, **Figure 6L** for overview). These results suggest enhanced KOR regulation in striatal *Pdyn* neurons during late adolescence, potentially tuning inhibitory control over striatal output.

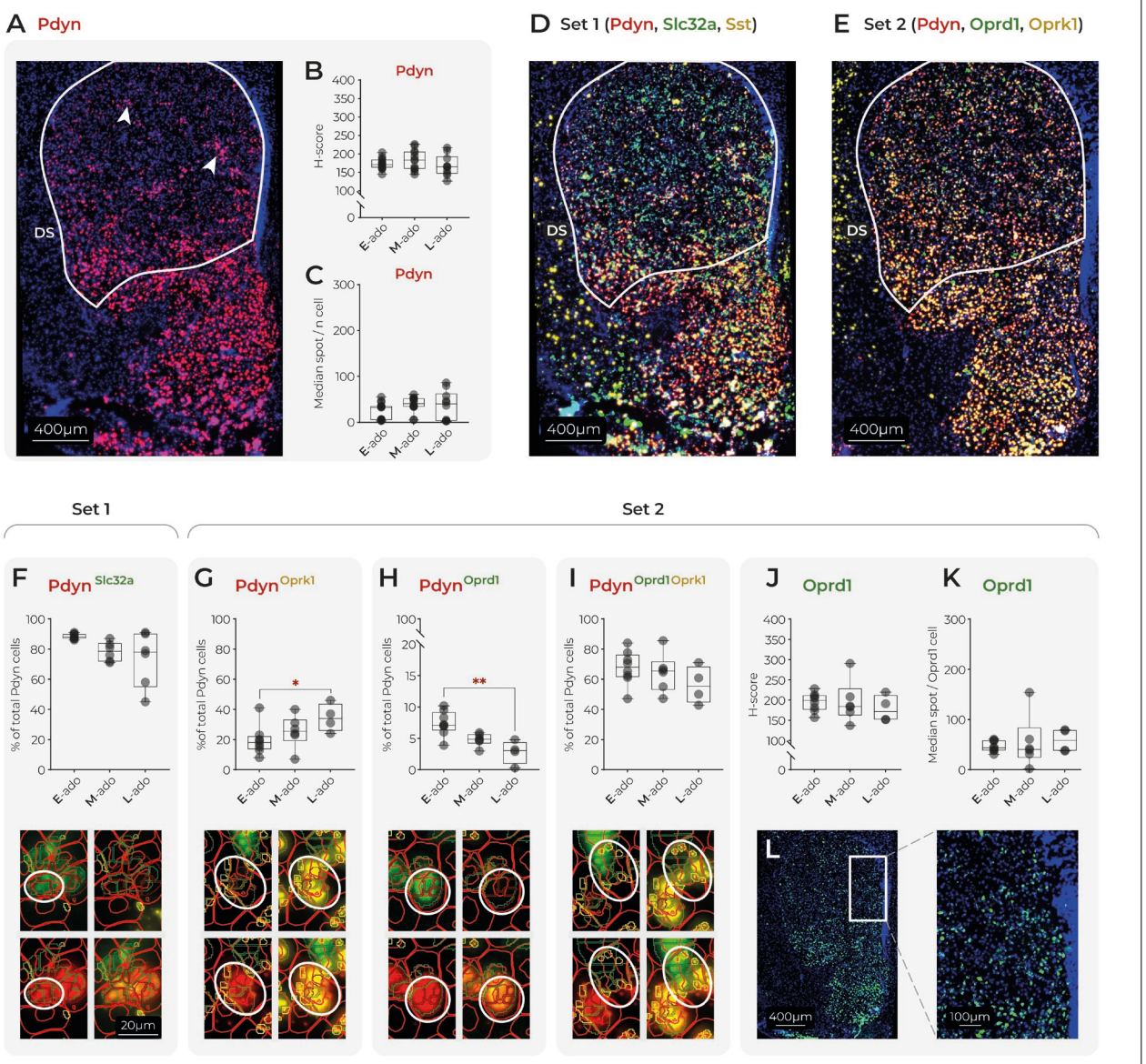

Figure 6. Developmental changes in *Pdyn*$^{+}$ populations in the DS

(A) Representative image of *Pdyn* mRNA (red) expression in the DS.
(B) *Pdyn* mRNA expression quantified using the H-score.
(C) Median number of *Pdyn* mRNA spots per *Pdyn*$^{+}$ cell.
(D) Representative images showing DS *Pdyn* (red), *Slc32a1* (green), and *Sst* (yellow) mRNA expression.
(E) Representative images showing DS *Pdyn* (red), *Oprd1* (green), and *Oprk1* (yellow) mRNA expression.
(F) Changes in the fraction of *Pdyn*$^{Slc32a+}$ neurons.
(G-I) Changes in the fraction of *Pdyn*$^{Oprk1+}$, *Pdyn*$^{Oprd1+}$ and *Pdyn*$^{Oprd1Oprk1+}$ neurons, respectively.
(J) *Oprd1* mRNA expression quantified using the H-score.
(K) Median number of *Oprd1* mRNA spots per *Pdyn*$^{+}$ cell.
(L) Representative images of *Oprd1* mRNA (green) expression in the DS.

Boxplots represent interquartile ranges with median values, and whiskers indicate the range. Each circle represents an individual animal. Statistical analyses were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test, with significance indicated as $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

For panels **(F–I)**, each graph is accompanied by an example cell indicated with a white circle. Cell nuclei, perimeters, and RNAscope signals were detected and annotated using QuPath software. Each set of four images shows the three individual channels and a merged image. Scale bars are shown in each image.

Nucleus accumbens

The NAc exhibited high levels of *Pdyn* mRNA, with the shell subregion showing the most abundant expression (**Figure 7A**). Similar to the DS, 83% of all *Pdyn*-positive cells were also found to have *Slc32a1* (**Figure 7F**). RNAscope revealed a decline in *Pdyn* transcript expression in the NAc in late- compared to early- and mid-adolescents, both in total signal (**Figure 7B**, H = 16.0, p = .0003) and per-cell transcript counts (**Figure 7C**, H = 9.05, p = .01). Notably, the population of $Pdyn^{Oprk1}$ neurons significantly increased in late- compared to early-adolescents (**Figure 7G**, H = 11.6, p = .0003) while populations of $Pdyn^{Oprd1}$ (**Figure 7H**, H = 7.0, p = .02) and the double-positive population neurons decreased (**Figure 7I**, H = 10.3, p = .001). This was accompanied by a reduction in total *Oprd1* transcript levels in early- relative to mid-adolescents (**Figure 7J,** H-score, H = 11.44, p = .004, **Figure 7K,** median spots per cell, H = 6.5, p = .03, **Figure 7L** for overview). In contrast, *Oprk1* expression remained stable across development, with no significant differences observed in either H-score or median spots per cell (both $p > 0.05$).

Interpretation of the RNAscope data requires caution when analyzing the green fluorescence channel (*Oprd1* and *Slc32a1* probes). The apparent decreases in total *Slc32a1* expression from early- to late-adolescence in both the DS not shown in the main body of results (H = 10.9, p = .0008) and the NAc (H = 6.1, p = .04) are biologically implausible, given that most neurons in these regions are GABAergic and there is no evidence for shifts in glia-to-neuron ratios (Saunders *et al.*, 2018; Anderson *et al.*, 2023). These observations likely reflect technical artifacts due to the small sample size and photobleaching, as green fluorophores are more sensitive to high-energy excitation light than red or yellow fluorophores, leading to faster signal decay. I report these results for completeness, even though this portion of the analysis requires replication. Accordingly, in the interpretation of the RNAscope data, I have focused on *Pdyn* co-localization with *Sst* and *Oprk1*, while total transcript abundance was primarily assessed using qPCR data.

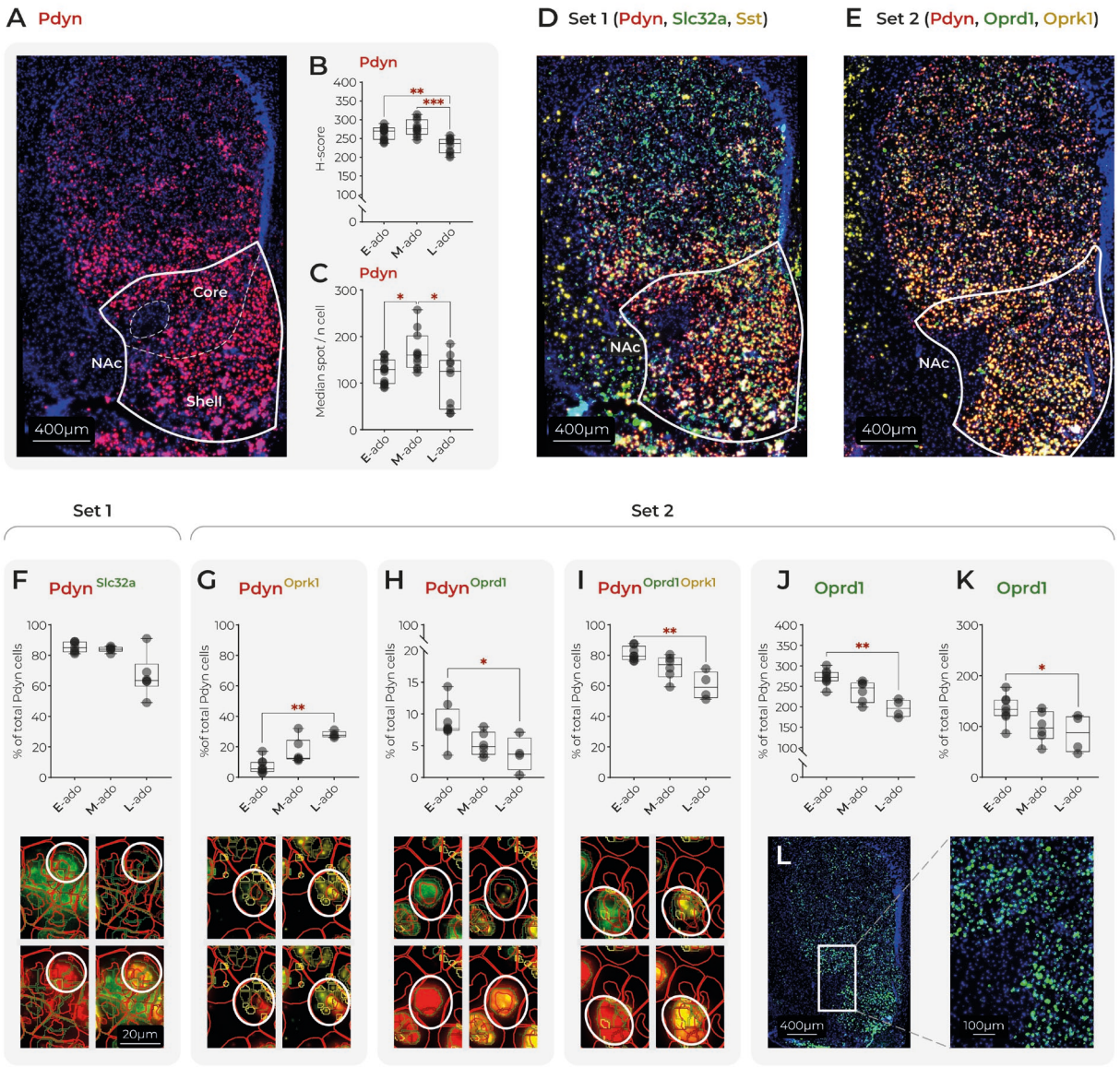

Figure 7. Developmental changes in *Pdyn*⁺ populations in the NAc

(A) Representative image of *Pdyn* mRNA (red) expression in the NAc.
(B) *Pdyn* mRNA expression quantified using the H-score.
(C) Median number of Pdyn mRNA spots per *Pdyn*⁺ cell.
(D) Representative images showing NAc *Pdyn* (red), *Slc32a1* (green), and *Sst* (yellow) mRNA expression.
(E) Representative images showing NAc *Pdyn* (red), *Oprd1* (green), and *Oprk1* (yellow) mRNA expression.
(F) Changes in the fraction of $Pdyn^{Slc32a^+}$ neurons.
(G-I) Changes in the fraction of $Pdyn^{Oprk1^+}$, $Pdyn^{Oprd1^+}$ and $Pdyn^{Oprd1Oprk1^+}$ neurons, respectively.
(J) *Oprd1* mRNA expression quantified using the H-score.
(K) Median number of *Oprd1* mRNA spots per *Pdyn*⁺ cell.
(L) Representative images of *Oprd1* mRNA (green) expression in the NAc.

Boxplots represent interquartile ranges with median values, and whiskers indicate the range. Each circle represents an individual animal. Statistical analyses were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test, with significance indicated as $p \leq 0.05$ (*) and $p \leq 0.01$ (**), $p \leq 0.001$ (***).

For panels **(F–I)**, each graph is accompanied by an example cell indicated with a white circle. Cell nuclei, perimeters, and RNAscope signals were detected and annotated using QuPath software. Each set of four images shows the three individual channels and a merged image. Scale bars are shown in each image.

Modulation of DYN/KOR signaling and reward behavior during adolescence

Transcriptional analyses revealed a decline in relative *Pdyn* expression between early- and mid-adolescence in both the mPFC and the DS, accompanied by an increase in the proportion of $Pdyn^{Oprk1+}$ neurons from early- to late-adolescence across the mPFC, the DS, and the NAc. This developmental shift in the DYN/KOR system within corticostriatal circuits suggested that attenuated DYN signaling may contribute to the reduced reward sensitivity characteristic of adolescence.

To test this hypothesis, I administered the selective, long-acting KOR antagonist norbinaltorphimine (norBNI; 10 mg/kg, 5 μL/g) immediately after the CPP pretest to block receptor activity throughout both the acquisition and expression phases of conditioning. I evaluated two types of reward, social interaction (**Figure 8A**) and cocaine (**Figure 9A**), to determine whether KOR involvement in CPP is reward-specific. All experiments employed a biased CPP design.

Comparison of the time spent in the social-paired context between pretest and posttest confirmed that conditioning significantly increased preference for the social-paired chamber in all groups (**Figure 8B**, Conditioning: $F_{(1.77)} = 100.0$, $p < .0001$). A significant Age × Treatment × Conditioning interaction ($F_{(2.77)} = 3.28$, $p = .043$) indicated potential age- and phase-dependent differences between norBNI- and vehicle-treated animals, despite the absence of main effects of Treatment ($F_{(1.77)} = .004$, $p = .949$) or Age ($F_{(2.77)} = .15$, $p = .859$). No other interactions reached significance (Age × Treatment $F_{(2.77)} = 2.81$, $p = .066$, Age × Conditioning, $F_{(2.\ 77)} = 1.38$, $p = .258$, Treatment × Conditioning, $F_{(1.77)} = .22$, $p = .639$). The significant age-dependent effect of norBNI administration was further supported when controlling for random differences in initial context preference, as indicated by the CPP index (posttest – pretest time spent in the social context; **Figure 8C**). Analysis of index revealed a significant Treatment × Age interaction ($F_{(2.77)} = 3.32$, $p = .042$), with no main effects of Treatment ($F_{(1.77)} = .21$, $p = .645$) or Age ($F_{(2.77)} = 1.39$, $p = .254$). Post hoc analysis indicated that KOR blockade selectively reduced social CPP in late-adolescent mice, whereas early- and mid-adolescent groups were unaffected.

Administration of norBNI did not influence general locomotor activity - all groups exhibited comparable increases in distance traveled from pretest to posttest. A main effect of Conditioning was observed on distance moved (**Figure 8D,** $F_{(1.77)} = 1.80$, $p = .183$), with no effects of Age (Age $F_{(2.77)} = .01$, $p = .989$)

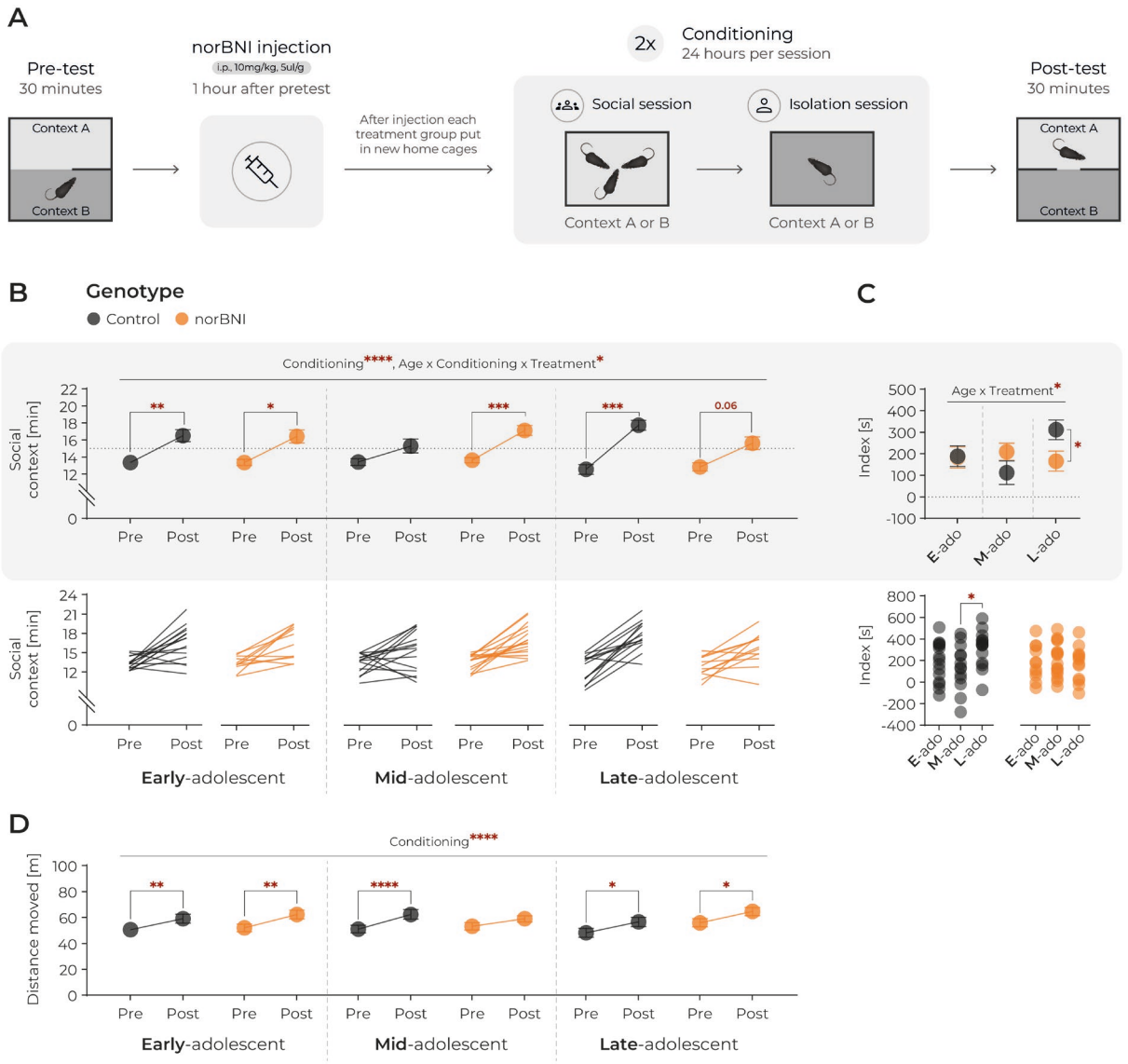

Figure 8. Selective KOR inhibition lowers social CPP in late-adolescent male mice

Control and norBNI-treated animals are shown in black and orange, respectively.

(A) Schematic timeline of the social CPP experiment, showing the timing of norBNI administration.

(B) Time spent in the social context during the pretest and posttest sessions. Upper panel: circles represent group means, and connecting lines represent matched values. Lower panels: each circle represents an individual animal. Whiskers indicate s.e.m. The dotted lines represent no change in preference (15 min). Statistical analysis was performed using a three-way ANOVA followed by Tukey's HSD. Significance levels are indicated as $p \leq .05$ (*), $p \leq .01$ (**), $p \leq .001$ (***), $p \leq .0001$ (****).

(C) Difference between time spent in the social context during posttest and pretest (CPP index). Upper panel: circles represent group means. Lower panels: each circle represents an individual animal. Whiskers indicate s.e.m. The dotted line on the y-axis (0) indicates no change in preference between sessions. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD. Significance is shown as $p \leq .05$ (*).

(D) Distance moved during the pretest and posttest sessions. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. Statistical analysis was performed using a three-way ANOVA followed by Tukey's HSD. Significance levels are indicated as $p \leq .05$ (*), $p \leq .01$ (**), $p \leq .0001$ (****).

or Age × Conditioning interaction (Age × Conditioning, $F_{(2.77)} = .09$, $p = .906$). Likewise, no significant effects or interactions involving Treatment were detected (Treatment $F_{(1.77)} = 1.80$, $p = .183$; Age × Treatment, $F_{(2.77)} = 1.13$, $p = .329$; Treatment × Conditioning $F_{(1.77)} = .350$, $p = .556$, Age × Treatment × Conditioning, $F_{(2.77)} = 1.56$, $p = .216$), confirming that social CPP was not confounded by locomotor activity.

Collectively, these findings suggest that KOR signaling facilitates social reward processing during late-adolescence, a developmental stage when *Pdyn* expression is reduced in cortical and dorsal striatal regions relative to early-adolescence. This apparent dissociation between *Pdyn* levels and KOR functional effects indicates that declining *Pdyn* expression may enhance network sensitivity to KOR modulation, potentially through altered intracellular signaling efficiency.

To test whether KOR's age-dependent effects extend to drug reward, cocaine CPP was assessed in adolescent mice (**Figure 9A**). Cocaine conditioning produced an increase in time spent in the reward-paired context across all groups (**Figure 9B**, $F_{(1.37)} = 64.51$, $p < .0001$). There was a significant effect of the interaction between Age × Conditioning on the time spent in the cocaine-paired context ($F_{(2.37)} = 7.22$, $p = .0023$), but no main effects of Treatment ($F_{(1.37)} = .001$, $p = .972$) or Age ($F_{(2.37)} = 2.37$, $p = .107$) and no other significant interactions (Age × Treatment, $F_{(2.37)} = 0.42$, $p = .663$, Treatment × Conditioning, $F_{(1.37)} = .36$, $= .549$, Age × Treatment × Conditioning, $F_{(2.37)} = .17$, $p = .843$). Thus, norBNI treatment did not affect cocaine CPP expression. Analysis of the CPP index confirmed the absence of a treatment effect (**Figure 9C**, Treatment $F_{(2.37)} = 7.17$, $p = .0023$) but revealed a significant main effect of Age ($F_{(2.37)} = 7.19$, $p = .002$) independent of Treatment (Treatment × Age $F_{(2.37)} = .17$, $p = .844$). Late-adolescent males exhibited significantly lower cocaine CPP compared to early- and mid-adolescents, irrespective of sex or norBNI treatment.

NorBNI had no effect on locomotor activity in the cocaine CPP test (**Figure 9D**). There were no significant effects of Treatment ($F_{(1.37)} = .079$, $p = .779$), or interactions involving treatment (Age × Treatment: $F_{(2.37)} = 3.11$, $p = .056$, Treatment × Conditioning: $F_{(1.37)} = 1.65$, $= .207$, Age × Treatment × Conditioning: $F_{(2.37)} = .65$, $p = .526$). Significant main effects of Conditioning ($F_{(1.37)} = 9.53$, $p = .004$)

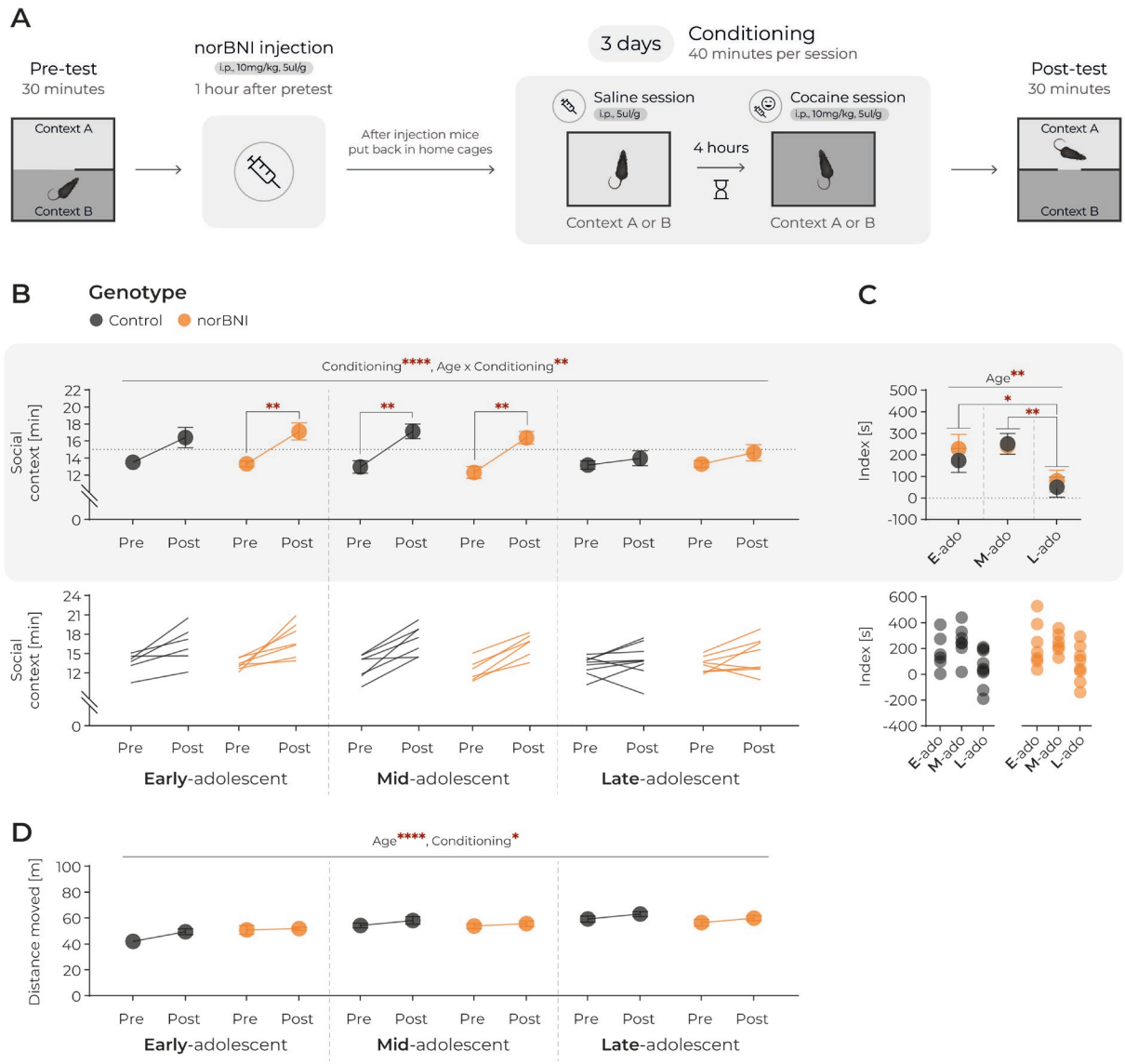

Figure 9. Selective KOR inhibition does not affect adolescent cocaine CPP

Control and norBNI-treated animals are shown in black and orange, respectively.

(A) Schematic timeline of the cocaine CPP experiment, showing the timing of norBNI administration.

(B) Time spent in the cocaine context during the pretest and posttest sessions. Upper panel: circles represent group means, and connecting lines represent matched values. Lower panels: each circle represents an individual animal. Whiskers indicate s.e.m. The dotted lines represent no change in preference (15 min). Statistical analysis was performed using a three-way ANOVA followed by Tukey's HSD. Significance levels are indicated as $p \leq .01$ (**), $p \leq .0001$ (****).

(C) Difference between time spent in the cocaine context during posttest and pretest (CPP index). Upper panel: circles represent group means. Lower panels: each circle represents an individual animal. Whiskers indicate s.e.m. The dotted line on the y-axis (0) indicates no change in preference between sessions. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD. Significance is indicated as $p \leq .05$ (*), $p \leq .01$ (**).

(D) Distance moved during the pretest and posttest sessions. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. Statistical analysis was performed using a three-way ANOVA followed by Tukey's HSD. Significance levels are indicated as $p \leq .05$ (*), $p \leq .0001$ (****).

and Age ($F_{(2.37)}$ = 19.20, p < .0001) on distance moved in pretest and posttest, indicating that generally mice increased distance moved during second test session ($F_{(1.77)}$ = 1.80, p = .183). No Age × Conditioning interaction was observed ($F_{(2.37)}$ = .09, p = .910). In contrast to the social paradigm, KOR blockade did not influence cocaine CPP at any developmental stage, indicating that KOR signaling selectively modulates social, but not drug-related, reinforcement during adolescent maturation.

Behavioral characterization of adult *Pdyn* KO mice

Finally, to verify the role of *Pdyn* in the regulation of social behaviors, I have used genetically modified mice with complete inactivation of the *Pdyn* gene (*Pdyn* KO). *Pdyn* transcript was absent in mutant mice, with no detectable expression in the striatum, where expression is normally abundant (**Figure 10A**).

First, I have performed a general assessment of the behavioral phenotype in mutant adult mice. Exploratory behavior was evaluated in the open-field test. The test was conducted under dim lighting to minimize anxiety-related effects, so that changes in behavior primarily reflect exploratory drive rather than anxiety. *Pdyn* KO mice spent a similar percentage of time in the center of the open field compared to control animals (41% vs. 43%, **Figure 10B,** Genotype: $F_{(1.31)}$ = .2988, p = .589). Females spent significantly less time in the center than males (38% vs. 47%), independent of genotype (Sex, $F_{(1.31)}$ = 7.43, p = .0104), with no significant Sex × Genotype interaction ($F_{(1.31)}$ = .03, p = .867). There was no significant effect of sex, genotype, or their interaction on total distance moved in the test (**Figure 10C**, Sex $F_{(1.31)}$ = .57, p = .4574, Genotype $F_{(1.31)}$ = .78, p = .3851, Sex × Genotype $F_{(1.31)}$ = .58, p = .451), indicating intact locomotor activity in *Pdyn* KO mice. Immediately afterward, a novel, age- and sex-matched conspecific was introduced into the experimental cage, allowing unrestricted social interaction. The primary measure was social interactions initiated by the subject mouse, including sniffing, grooming, chasing, and mounting, which reflect behaviors expressed in proximity to the stimulus animal. *Pdyn* KO mice exhibited similar levels of sociability as control animals, with no main effect of genotype (**Figure 10D**, Genotype: $F_{(1.32)}$ = .6078, p = .4413) and no significant Sex × Genotype interaction ($F_{(1.32)}$ = .14, p = .7083). Females, however, engaged in significantly more social interaction than males (195 s vs. 97 s), independent of genotype (Sex: $F_{(1.32)}$ = 29.45, p < .0001).

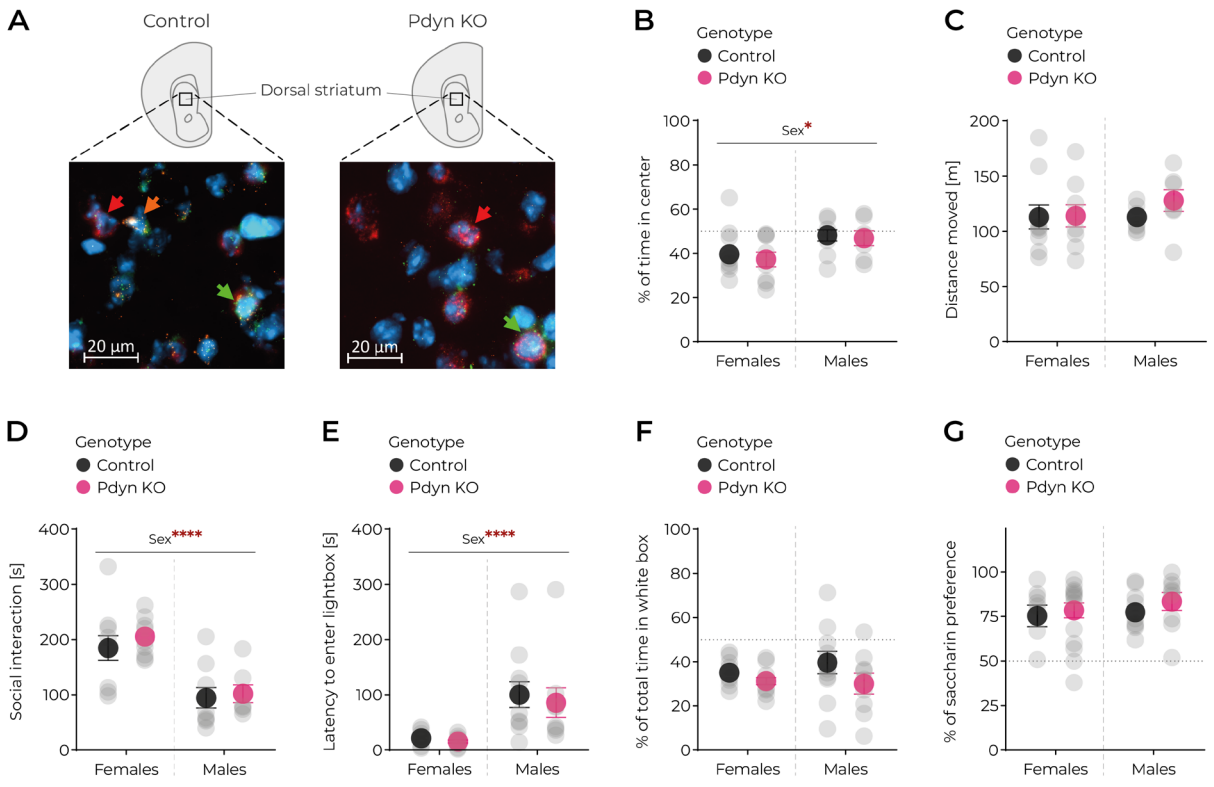

Figure 10. Adult *Pdyn* KO mice display intact exploratory behavior, normal anxiety-like responses, preserved social behavior, and no anhedonia-like behavior.

Control and *Pdyn* KO animals are shown in black and magenta, respectively.

(A) Representative RNAscope *in situ* hybridization images in the dorsal striatum (DS) of control and *Pdyn* KO mice. *DAPI* (blue), *Pdyn* (Atto550, orange), *Penk* (Atto647, red), and *Oprd1* (Alexa Fluor 488, green) mRNA expression is shown. Arrows indicate example transcript sites; arrow color corresponds to the fluorophore described above. Scale bars are shown in each image.

(B–D) Exploratory and social behavior in the open field.
(B) Percentage of time spent in the center of the open field.
(C) Total distance moved [m].
(D) Time spent in social interaction [s].

Circles represent group means (black/magenta) and individual animals (gray). Whiskers indicate s.e.m. The dotted line denotes the chance level (50%). Statistical analysis was performed using two-way ANOVA followed by Tukey's HSD. Significance is indicated as $p \leq .05$ (*) and $p \leq .0001$ (***).

(E–F) Anxiety-like behavior in the light-dark box.
(E) Latency to enter the light compartment [s].
(F) Percentage of time spent in the light compartment.
Circles represent group means (black/magenta) and individual animals (gray). Whiskers indicate s.e.m. The dotted line denotes the chance level (50%). Statistical analysis was performed using two-way ANOVA followed by Tukey's HSD. Significance is indicated as $p \leq .0001$ (****).

(G) Anhedonia symptoms were measured in saccharin preference task. Circles represent group means (black/magenta) and individual animals (gray). Whiskers indicate s.e.m. The dotted line denotes chance level (50%). Statistical analysis was performed using two-way ANOVA.

Next, I assessed anxiety-like behavior using the light-dark box test, which measures rodents' avoidance of bright, open spaces as an indicator of anxiety. Animals were placed in the unlit compartment, and both the latency to first entry of the light compartment and the total time spent in the light compartment were measured. There was no significant effect of Genotype or Sex × Genotype interaction on latency to enter the light compartment (**Figure 10E**, Genotype: $F_{(1.38)} = .37$, $p = .547$, Sex × Genotype: $F_{(1.38)} = .06$, $p = .8120$) or on total time spent in the light compartment (**Figure 10F**, Genotype: $F(1.38) = 3.45$, $p = .071$, Sex × genotype: $F_{(1.38)} = .62$, $p = .435$). However, there was a significant main effect of Sex on latency to enter the light compartment (**Figure 10E**, Sex: $F_{(1.38)} = 19.22$, $p < .0001$), with females entering the light box significantly faster than males, independent of genotype (18 s vs. 94 s delay). No sex differences were observed for total time spent in the light compartment (**Figure 10F**, Sex: $F_{(1.38)} = .22$, $p = .638$), with all groups spending an average of 34% of the session in the light compartment. These results indicate that, although females initiate entry into the light compartment more quickly than males, overall time spent in the light was similar across sex and genotype.

DYN signaling is involved in aversion and mood regulation (Merrer *et al.*, 2009; Bruchas *et al.*, 2010; Knoll & Carlezon, 2010). To evaluate a potential anhedonia-like phenotype, mutant mice were tested for their preference for sweet taste. Preference for a saccharin-sweetened solution versus plain water was measured, as rodents typically find saccharin highly palatable despite its lack of calories. Reduced preference indicates anhedonia. All animals exhibited high saccharin preference, irrespective of sex or genotype (**Figure 10G**, Sex × Genotype: $F_{(1.41)} = .10$, $p = .752$, Sex: $F_{(1.41)} = .54$, $p = .4677$, Genotype: $F_{(1.41)} = .91$, $p = .347$), indicating intact hedonic perception in *Pdyn* KO mice.

To account for potential differences in the development of C57BL/6 and *Pdyn* KO mice, I measured markers of puberty, including testis weight, serum testosterone, and preputial separation (**Figure 11**). Serum testosterone was measured in trunk blood from mice used also in RNAscope experiments (**Table 2**) by Lab-Wet (Kraków, Poland).

While testis weight increased with age, no significant age-related changes in serum testosterone were observed (**Figure 11A**), suggesting that a testosterone surge may not have occurred yet or that the assay

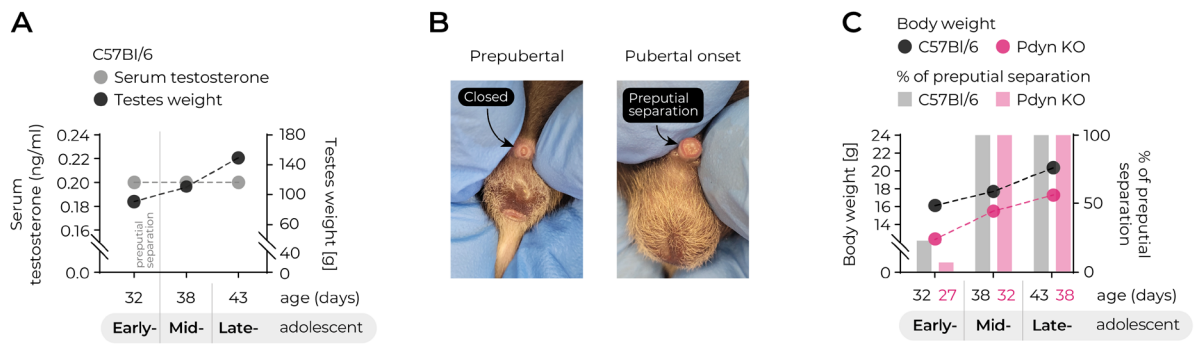

Figure 11. Pubertal markers in adolescent male mice.

(A) Testis weight and serum testosterone levels in C57BL/6 mice bred at IF PAN. Left *y*-axis: serum testosterone (ng/ml; gray circles). Right *y*-axis: testis weight (g; black circles). Dashed lines represent the schematic developmental trajectory of each parameter. The vertical gray line indicates the age point at which preputial separation is completed.

(B) Representative image illustrating preputial separation, showing detachment of the prepuce from the glans penis.

(C) Comparison of body weight and incidence of preputial separation in C57BL/6 and wild-type *Pdyn* KO mice. Shades of gray represent C57BL/6 mice, and shades of pink represent wild-type *Pdyn* KO mice. Left *y*-axis: body weight (g; circles). Right *y*-axis: percentage of males exhibiting preputial separation (bars). Dashed lines represent the schematic developmental trajectory of body weight.

was insufficiently sensitive to detect small fluctuations. I have also evaluated preputial separation that was reported to precede the increase in testosterone levels (Bell, 2018, **Figure 11B**). In the C57BL/6 cohort, 27% of early-adolescent males showed preputial separation, reaching 100% by mid-adolescence (**Figure 11C**). Since the timing of preputial separation is strain-dependent, it was used to align adolescent stages in *Pdyn* KO males. Despite P33 *Pdyn* KO males being lighter than P32 C57BL/6 males (mean ± SEM: 14 ± 1.3 g vs. 18 ± 0.2 g; **Table 4&6**), all P33 *Pdyn* KO males had complete preputial separation, consistent across genotypes. Accordingly, adolescent time points for *Pdyn* KO males were shifted approximately five days earlier to match developmental stages across strains.

Late-adolescent *Pdyn* KO males express reduced social CPP

Social CPP was assessed in *Pdyn* KO mice using the same protocol described previously (**Figure 12A**). As expected, pre-post analysis revealed a significant effect of Conditioning on time spent in the social-paired context (**Figure 12B**, $F_{(1.66)} = 34.11$, $p < .0001$) without a significant Age × Conditioning interaction ($F_{(2.66)} = .07$, $p = .932$), and a trend of Genotype × Conditioning interaction ($F_{(1.66)} = 3.8$, $p = .055$). A significant main effect of Genotype ($F_{(1.66)} = 5.88$, $p = .018$) and an Age × Genotype interaction ($F_{(2.66)} = 3.31$, $p = .043$) were observed. Age alone had no significant effect ($F_{(2.66)} = .07$, $p = .927$) on

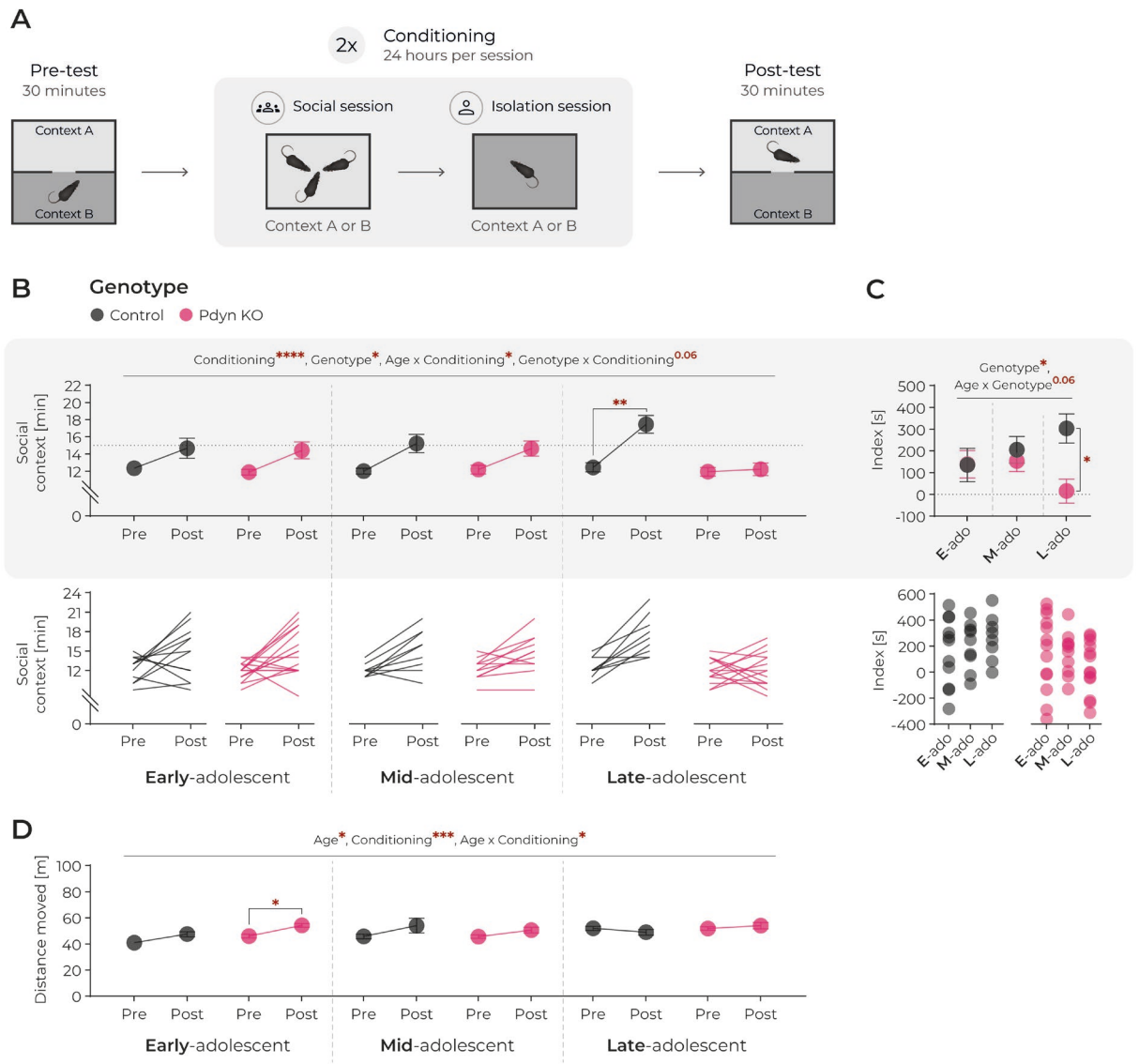

Figure 12. Late-adolescent *Pdyn* KO males recapitulate the reduction of social CPP by norBNI

Control and *Pdyn* KO animals are shown in black and magenta, respectively.

(A) Schematic timeline of the social CPP experiment with *Pdyn* KO mice.

(B) Time spent in the social context during the pretest and posttest sessions. Upper panel: circles represent group means, and connecting lines represent matched values. Lower panels: each circle represents an individual animal. Whiskers indicate s.e.m. The dotted lines represent no change in preference level (15 min). Statistical analysis was performed using a three-way ANOVA followed by Tukey's HSD. Significance levels are indicated as $p \leq .05$ (*), $p \leq .01$ (**), $p \leq .0001$ (****).

(C) Difference between time spent in the social context during posttest and pretest (CPP index). Upper panel: circles represent group means. Lower panels: each circle represents an individual animal. Whiskers indicate s.e.m. The dotted line on the y-axis (0) indicates no change in preference between sessions. Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD. Significance is indicated as $p \leq .05$ (*).

(D) Distance moved during the pretest and posttest sessions. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. Statistical analysis was performed using a three-way ANOVA followed by Tukey's HSD. Significance levels are indicated as $p \leq .05$ (*), $p \leq .001$ (***).

change in time spent on social context from pretest to posttest. A trend toward a triple interaction (Age × Genotype × Conditioning $F_{(2.66)}$ = 2.84, p = .065) further suggested age-dependent effects of *Pdyn* deletion. Analysis of the CPP index confirmed a significant effect of Genotype (**Figure 12C**, $F_{(1.66)}$ = 4.34, p = .041) and showed a trend toward an Age × Genotype interaction ($F_{(2.66)}$ = 2.84, p = .066), with no main effect of Age (Age $F_{(2.66)}$ = .212, p = .806) on expression of social CPP. These findings indicate that *Pdyn* KO mice generally exhibited lower social CPP, with the reduction most pronounced in late adolescence. These were no significant effect of Genotype or Genotype × Conditioning interaction on changes in distance moved from pretest to posttest (**Figure 12D,** Age, $F_{(2.66)}$ = 3.69, p = .0302, Genotype $F_{(1.66)}$ = 2.49, p = .119, Conditioning $F_{(1.66)}$ = 15.56, p = .0002, Age × Genotype, $F_{(2.66)}$ = 2.55, p = .085, Age × Conditioning $F_{(2.66)}$ = 4.76, = .012, Genotype × Conditioning $F_{(1.66)}$ = .27, p = .603, Age × Genotype × Conditioning, $F_{(2.66)}$ = 1.03, p = .361).

Overall, late-adolescent *Pdyn* KO mice recapitulated the phenotype observed with pharmacological KOR blockade: social CPP was selectively reduced during late-adolescence, while earlier developmental stages remained unaffected. These results demonstrate that both genetic loss of *Pdyn* and KOR antagonism impair social reward learning specifically during late adolescence.

DYN/KOR signaling is essential for social, but not cocaine CPP in late-adolescent mice

To integrate these findings, I compared the effects of DYN/KOR modulation (via norBNI or *Pdyn* KO) across social and cocaine CPP using normalized z-scores of preference indices (**Figure 13**). In social CPP experiments, control groups (vehicle/saline) did not differ statistically (t-test, p > .05) and were pooled to reduce complexity. The z-score index reflects CPP expression relative to the overall mean across all three experiments (social + norBNI, cocaine + norBNI, and social *Pdyn* KO). Importantly, this analysis was intended to compare the effects of DYN/KOR modulation within each reward type, not to directly compare the magnitude of social versus cocaine CPP, as differences in motivational value between reward types require concurrent conditioning and careful control of parameters such as dose and session timing (discussed in Misiołek *et al.*, 2025, Section **10.2** *Original paper 2*). Analysis of social CPP revealed a significant main effect of DYN/KOR modulation and an Age × DYN/KOR interaction on z-score normalized indexes (**Figure 13A**), DYN/KOR: $F_{(2.19)}$ = 4.32, p = .015,

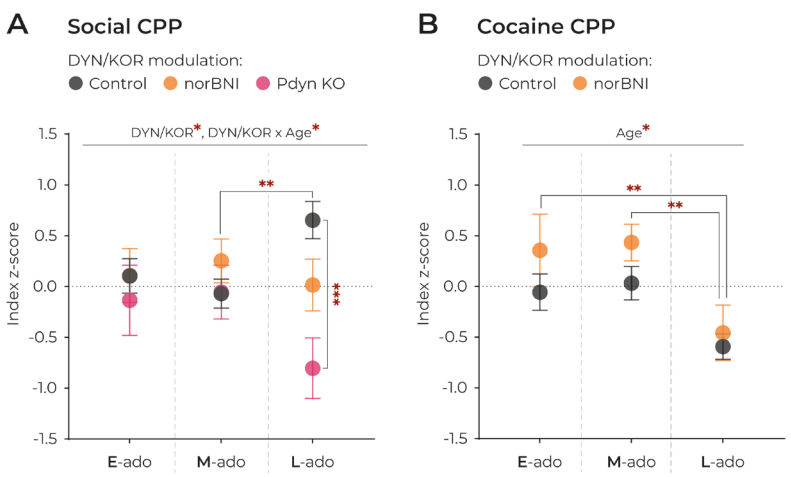

Figure 13. Late-adolescent *Pdyn* KO males recapitulate the reduction of social CPP by norBNI

Control, norBNI, and *Pdyn* KO animals are shown in black, orange, and magenta, respectively. Control groups (vehicle and saline) were combined. The index z-score was calculated across all CPP experiments as: (individual animal CPP index – mean CPP index of all mice) / standard deviation of CPP index of all mice.

(A) Relative social CPP in control, norBNI-treated, and *Pdyn* KO males. Circles represent group means, and whiskers correspond to s.e.m. The dotted lines represent no change in preference level. Statistical analysis was performed using 2-way ANOVA followed by Tukey's HSD. Significance is indicated as $p \leq .05$ (*), $p \leq .01$ (**), $p \leq .001$ (***).

(B) Relative cocaine CPP in control and *Pdyn* KO males. Circles represent group means, and whiskers correspond to s.e.m. The dotted lines represent no change in preference level. Statistical analysis was performed using 2-way ANOVA followed by Tukey's HSD. Significance is indicated as $p \leq .05$ (*), $p \leq .01$ (**).

Age × DYN/KOR: $F_{(4.19)} = 3.083$, $p = .0172$), but no main effect of Age alone ($F_{(2.19)} = .09$, $p = .908$). Control late-adolescent mice expressed higher social CPP than mid-adolescents, whereas *Pdyn* KO late-adolescents exhibited significantly reduced social CPP relative to late-adolescent controls. In contrast, cocaine CPP was influenced solely by Age (**Figure 13B**, $F_{(2.69)} = 7.924$, $p = .0008$), with no effect of DYN/KOR modulation ($F_{(1.69)} = 3.21$, $p = .077$), or DYN/KOR × Age interaction ($F_{(2.69)} = .28$, $p = .079$). Late-adolescent males expressed significantly lower cocaine CPP than early- and mid-adolescents, independent of DYN/KOR modulation.

Overall, these findings show that DYN/KOR signaling selectively regulates social, but not drug-related reward during late-adolescence. Both pharmacological KOR blockade and genetic deletion of *Pdyn* reduce social CPP specifically in late-adolescent mice, highlighting an age-dependent role of this system. This work underscores the importance of developmental changes in DYN/KOR circuits for the maturation of social motivation and reward sensitivity.

7. Discussion

7.1 A behavioral paradigm for measuring social reward in mice

My goal was to investigate the behavioral and neurobiological mechanisms underlying social interactions with peers. I examined how reward processing evolves across adolescence, with a particular focus on social reward and its interaction with endogenous opioid signaling. My results reveal that basal reward sensitivity, particularly for social reward, is reduced during adolescence, and gradually becomes dependent on DYN/KOR signaling following sexual maturation.

I first characterized prosocial behavior, social reward, and affective state discrimination in adult C57BL/6 mice (Misiołek *et al.*, 2023, Section **10.1** *Original paper 1*). This initial characterization provided two key insights. Females showed stronger prosocial tendencies than males, sharing rewards with familiar peers (*Original paper 1,* **Figure 1**). Both sexes showed comparable sensitivity to social reward in the social CPP task (*Original paper 1,* **Figure 2**) and displayed similar abilities to discriminate a familiar partner with an altered affective state from a partner with a neutral, unchanged affective state (*Original paper 1,* **Figure 3**). This contrasts with observations by Scheggia and collaborators, who reported that male mice were more likely than females to perform prosocial actions in a similar food-sharing task (Scheggia *et al.*, 2022). Previously reported experiments were conducted in an automated operant chamber that allowed for direct tactile contact between the actor and the partner. In contrast, our apparatus was manually operated and separated the animals with a transparent but non-perforated partition, restricting physical contact. These methodological differences may explain the discrepancy between our results and highlighting the influence of experimental design on observed sex-specific behavior. Importantly, my study also demonstrated that adult mice of both sexes display robust social CPP when more conditioning sessions are performed and the social context involves familiar siblings. This finding extends our previous report (Harda *et al.*, 2022), which demonstrated the same effect in adult females, and together these results reconcile prior discrepancies in the literature regarding the expression of social CPP in adult mice (Nardou *et al.*, 2019).

Having established that adult mice find same-sex, non-reproductive social interactions rewarding, I then asked when and how these mechanisms emerge. Early-life sociality is dominated by kin-based interactions, but as individuals enter adolescence, interactions shift toward non-kin and, ultimately, sexually motivated exchanges (Panksepp *et al.*, 2007; Varlinskaya & Spear, 2008; Pellis & Pellis, 2017). This transition reflects a reorganization of social motivation, during which neural circuits governing social behavior are refined to support more flexible and context-dependent roles. To investigate this process, I extended the social CPP task to capture the evolution of the motivational value of peer-social interactions across three stages of adolescence in C57BL/6 mice: early (pubertal onset), mid (peripubertal), and late (sexual maturity) in both sexes. By including parallel CPP experiments with palatable food as a different natural reward, and cocaine as a drug reward, I aimed to determine whether developmental changes in reward processing were specific to the social domain (Misiołek *et al.*, 2025, Section **10.2** *Original paper 2*).

Although adolescence is generally regarded as a period of increased reward sensitivity (Spear, 2000, 2013; Knudsen, 2004, 2004; Galván, 2010, 2013; Walker *et al.*, 2017), I found that adolescent mice of both sexes displayed lower CPP than adults when all reward types were analyzed together (*Original paper 2,* **Figure 4**). This developmental profile could not be attributed to confounding factors such as differences in locomotor activity, learning impairment, or increased novelty seeking. I hypothesize that reward sensitivity during adolescence is context-dependent - heightened primarily in emotionally salient or stressful contexts, but reduced under low-stress conditions (Spear, 2000, 2013; Poon, 2017). I conducted all tests under minimal stress conditions, thus, the attenuated CPP across social, food, and drug rewards likely reflects a general developmental reduction in reward responsiveness. I also examined the variability of CPP within adolescence. When preference was assessed based on the proportion of individuals showing higher or lower normalized CPP, developmental differences emerged. Social (*Original paper 2,* **Figure 1**) and food reward (*Original paper 2,* **Figure 2**) expression varied across adolescent stages, revealing a qualitative shift in CPP, whereas sensitivity to cocaine reward remained stable (*Original paper 2,* **Figure 3**). These findings align with previous reports showing a selective decrease in social, but not cocaine, CPP during adolescence, suggesting that natural rewards

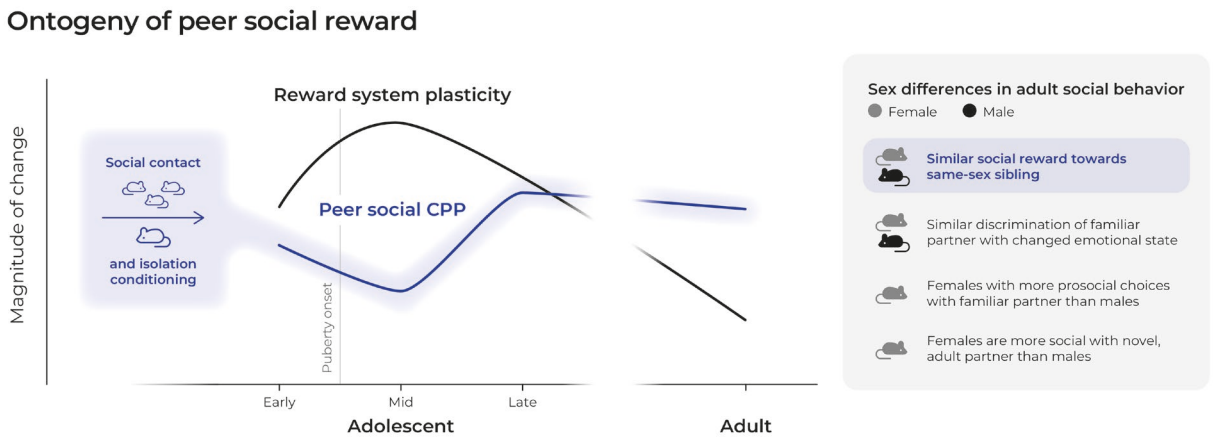

Figure 14. Adolescent mice show decreased peer-social reward place preference compared to adults.

Schematic illustration of the developmental trajectory of peer-social conditioned place preference (CPP) in mice (based on results from this PhD thesis) alongside the plasticity of the reward system (adapted and modified from Uhlhaas et al., 2023). The blue curve depicts developmental changes in social CPP, based on an extended conditioning paradigm that reliably induces social CPP in adults. The final session preceding the post-test represents a period of social isolation. The blue shaded area encompasses all data related to social CPP. The accompanying table summarizes sex differences characteristic of social behavior in adult C57BL/6 mice. The black curve represents the relative plastic potential of reward-related neural circuits, which peaks during adolescence.

may exhibit greater developmental dynamics than drug rewards during this period (Cann *et al.*, 2020; Harda *et al.*, 2025).

Together, these findings indicate that adolescent and adult mice of both sexes show strong sensitivity to social stimuli, providing a behavioral framework for investigating the neural basis of social reward and motivation (**Figure 14**). Adolescence may be marked by a general reduction in reward responsiveness, which likely reflects transient recalibration of motivational circuitry during a period of heightened plasticity in the corticolimbic reward system, when neural responses are particularly sensitive to experience-driven shaping (**Figure 14**).

7.2 Remodeling of corticolimbic opioid circuits during adolescence

Reward-related behaviors are orchestrated by the mesocorticolimbic dopamine system. Development of the dopaminergic system during adolescence has been studied extensively. It involves increased dopamine release capacity, growth of dopaminergic innervation, changes in expression of D1- and D2-like dopamine receptors, and enhanced responsiveness to salient stimuli (Andersen *et al.*, 1997;

Brenhouse *et al.*, 2008; Hoops *et al.*, 2018; Reynolds & Flores, 2021; Avramescu *et al.*, 2024). Conversely, the developmental trajectory of the endogenous opioid system, which shapes hedonic experiences, stress reactivity, and social bonding, has received much less attention (MERRER *et al.*, 2009; Inagaki, 2018; Galiza Soares *et al.*, 2025; Spodnick *et al.*, 2025). Previous reports on developmental changes in the endogenous opioid system have predominantly focused on prenatal, neonatal, early juvenile, or adult stages, leaving adolescence largely unexplored (Thorpe *et al.*, 2020; Spodnick *et al.*, 2025). In the context of reward behavior development, adolescence is likely a critical period: correlating changes in endogenous opioid activity with shifts in CPP could reveal not only how reward learning changes ontogenetically but also identify potential opioid targets that may predispose to affective and motivational disorders.

I examined developmental changes in opioid gene expression in corticolimbic regions: the mPFC, the NAc, and the DS, which are central to both reward processing and social behavior. I used qPCR to quantify two opioid peptide precursor transcripts, *Pdyn* and *Penk*, and opioid receptor mRNAs *Oprk1*, *Oprm1*, and *Oprd1*, in male C57BL/6 mice at early (~P32), mid (~P38), and late (~P43) adolescence. The results revealed a decrease in *Pdyn* expression from early- to mid-adolescence in both the mPFC and the DS, whereas *Pdyn* levels in the NAc remained stable (Section 6.2, **Figure 2**). To the best of my knowledge, no prior studies have described a similar developmental trajectory for *Pdyn* expression. Therefore, the present findings may represent the first evidence of a peripubertal, adolescence-specific downregulation of *Pdyn* expression in the mPFC–DS circuits. In contrast, expression of *Penk*, remained stable across all ages and structures examined (Section 6.2, **Figure 2**). Previously, Ellgren and collaborators reported that Met-enkephalin peptide levels in the NAc core of rats transiently decreased during mid-adolescence (P38) compared to both early (P29) and late (P50) adolescence, but differences in age, species, and measurement (peptide vs. mRNA) complicate direct comparison (Ellgren *et al.*, 2008). Overall, these data suggest DYN-, but not enkephalin-mediated, signaling may undergo substantial region-specific reorganization during adolescence.

Opioid receptor expression remained largely stable across age groups (Section 6.2, **Figure 2**). Neither *Oprk1* nor *Oprm1* showed significant age-dependent changes in the mPFC, the DS or the NAc,

consistent with earlier studies reporting relatively constant receptor densities throughout adolescence (Kornblum *et al.*, 1987; Ellgren *et al.*, 2008). It was also observed that MOR protein levels in the rat PFC increase from early- to mid-adolescence and subsequently decline, while KOR expression remained largely constant (Kornblum *et al.*, 1987). In case of DOR, previous radioligand binding studies reported higher DOR density in the rat PFC and the NAc during mid-adolescence compared to pre-adolescence (Kornblum *et al.*, 1987). In my study, *Oprd1* expression increased with age in the NAc but not in the PFC, partially supporting the earlier findings.

Taken together, these results point to region-specific changes in *Pdyn* gene expression across adolescence. Previous studies show that KOR activation, either via agonists administered into the NAc or the mPFC, or via DYN release in the dorsal medial striatum, produced aversion (Bals-Kubik *et al.*, 1993; Gowrishankar *et al.*, 2024). This underscores the functional engagement of DYN/KOR signaling in these circuits and the relevance of studying its developmental regulation. To identify which neuronal populations drive these transcriptional shifts, I complemented qPCR with RNAscope *in situ* hybridization, mapping *Pdyn* expression in relation to cell-type markers across the mPFC, the DS, and the NAc. This approach provided single-cell resolution, revealing the spatial and cellular context of developmental change that qPCR alone cannot resolve. qPCR analysis in homogenized brain tissue is robust in assessing mRNA abundance, but offers no insight into cell-type distribution of the transcript (Atout *et al.*, 2022), while RNAscope resolves spatial and cell-type heterogeneity, but the signal may be influenced by tissue preparation (Ariotta *et al.*, 2024). Previous studies show that *Pdyn* mRNA in striatal regions varies with anterior-posterior position (Torres-Reveron *et al.*, 2007). Thus, as RNAscope analysis was performed only on selected sections, the observed discrepancies between methods (e.g., different expression of *Pdyn* in NAc, qPCR vs. RNAscope) likely reflect resolution differences. I therefore consider the approaches to complement each other: qPCR captures region-wide expression trends, whereas RNAscope identifies local patterns and cell-type specificity.

The fraction of $Pdyn^{Oprk1+}$ neurons increased during adolescence across the mPFC (Section 6.2, **Figure 5**), the DS (Section 6.2, **Figure 6**), and the NAc (Section 6.2, **Figure 7**). In the mPFC, a transient peak was observed from early- to mid-adolescence, followed by a decrease in late adolescence (Section 6.2,

Figure 5). Previous literature indicates that *Pdyn* and *Oprk1* in the mPFC do not overlap, with *Pdyn*-positive neurons localized in superficial, and KOR is in deeper cortical layers (Yarur *et al.*, 2023). Given that co-expression of both transcripts remained under 5% in early and late adolescence (Section 6.2, **Figure 5**), this apparent mid-adolescent increase likely reflects a methodological artifact, such as overexposure during imaging. In contrast, co-expression of *Pdyn* and *Oprk1* within the DS (Section 6.2, **Figure 6**) and the NAc (Section 6.2, **Figure 7**) increased steadily with age. Striatal KORs are located on axon terminals on the same (DYN-expressing) neurons where they inhibit neurotransmitter release when stimulated (Tejeda *et al.*, 2017). Thus, the increasing overlap of *Oprk1* expression with that of *Pdyn* (coding for KOR agonists) may represent the gradual emergence of a tighter autoregulatory feedback, enabling finer control over DYN release via presynaptic KOR. Functionally, this could indicate that as the DYN/KOR system matures, it becomes less tonically active but more contextually responsive, requiring stronger input to engage.

Furthermore, the proportion of $Pdyn^{Sst+}$ interneurons increased from early to mid-adolescence in the mPFC (Section 6.2, **Figure 5**). The growing population of $Pdyn^{Sst+}$ interneurons likely contributes to top-down inhibitory control over stress responses. Recent work shows that such interneurons are activated by threats and release DYN, with the net effect of amplifying functional output in circuits that suppress passive defensive behaviors while promoting active coping strategies (Wang *et al.*, 2024). Speculatively, this shift could signify a developmental transition toward more proactive stress coping during late adolescence.

However, my interpretation refers to *basal* (unstimulated) *Pdyn* expression measured under resting conditions, reflecting the developmental state of the system rather than its functional activity. Importantly, *Pdyn* mRNA represents only the potential for DYN synthesis and does not necessarily indicate peptide release, receptor activation, or downstream signaling. Activity of the DYN/KOR system is highly context-dependent: its activity can shift in response to acute or chronic stress, social isolation, or drug exposure, none of which are captured by baseline transcript levels. For example, *Pdyn* expression can increase transiently after acute stress or drug administration, while chronic or repeated stress produces more complex, region-specific adaptations (McLaughlin *et al.*, 2003; Land *et al.*, 2008;

Carlezon & Thomas, 2009). Thus, while baseline *Pdyn* expression provides valuable insight into the developmental trajectory of the DYN/KOR system, it should not be interpreted as a direct readout of system function. In line with this, cocaine-induced CPA is associated with reduced *Pdyn* expression specifically in the ventral striatum, while dorsal striatal *Pdyn* remains unchanged (Nicot *et al.*, 2025). Moreover, animals with higher overall striatal *Pdyn* levels show reduced sensitivity to cocaine's aversive effects. Adding further complexity, the direction of KOR-mediated effects depends on developmental stage. Early-life or juvenile stress has mixed effects on KOR function - it can enhance, weaken, or not alter it (Karkhanis *et al.*, 2016; Diaz *et al.*, 2018; Lutz *et al.*, 2018), whereas stress in adults generally enhances DYN/KOR signaling (Knoll & Carlezon, 2010; Karkhanis *et al.*, 2017). Therefore, without corresponding behavioral or functional readouts such as stress reactivity, social motivation, or reward learning, it is not possible to determine whether the observed molecular changes reflect adaptive changes, altered stress responsivity, or a shift in the rewarding versus aversive balance of the reward system. Viewed in a broader context, these molecular observations likely represent elements of a coordinated developmental tuning process, in which the DYN/KOR system gradually shifts from diffuse to context-specific control of motivational states.

A broader developmental pattern becomes apparent when considering changes in *Pdyn* expression and *Pdyn/Oprk1* colocalization. Decreasing basal *Pdyn* levels coupled with increasing *Pdyn*/*Oprk1* colocalization may reflect a shift from a diffuse, easily activated network to a more selective, context-dependent system, tuned to respond under emotionally salient conditions. This interpretation aligns with developmental models of dopamine function - adolescence is characterized by low tonic but high phasic dopamine activity (Adriani & Laviola, 2003; Tirelli *et al.*, 2003), supporting learning that prioritizes emotionally meaningful experiences (Sturman & Moghaddam, 2011; Galván, 2013). Altered general reward sensitivity could lead to maladaptive changes (Wahlstrom *et al.*, 2010; Fairchild, 2011), but also may serve as an adaptive gating mechanism, limiting unnecessary plasticity. This proposed tuning of the DYN/KOR system suggests that molecular and circuit-level maturation during adolescence should be reflected in parallel shifts in reward-related motivation and behavior.

7.3 Developmental transitions in reward-related motivation

Behaviorally, CPP for rewards increased from adolescence to adulthood (Misiołek *et al.*, 2025, Section **10.2** *Original paper 2*). This age-related increase in reward preference occurred in parallel with a decrease in baseline *Pdyn* expression from early- to mid-adolescence (**Figure 15A**), suggesting an inverse relationship between *Pdyn* levels and reward sensitivity. At first glance, this seems straightforward: DYNs mediate aversive states, so lower basal *Pdyn* expression (and speculatively lower peptide secretion), would reduce inhibition of dopamine signaling, thereby enhancing reward-driven behavior. Such an interpretation assumes uniform regulation across all reward modalities. The social CPP is dynamic during adolescence, with pronounced individual variability and a decrease in mid-adolescence, whereas cocaine CPP remains relatively stable across age (Cann *et al.*, 2020; Harda *et al.*, 2025; Misiołek *et al.*, 2025). This discrepancy raises a crucial question: do CPP paradigms across different reward types measure the same construct of "*reward*" or rather distinct motivational processes?

CPP integrates multiple components: when a mouse approaches or avoids a context paired with a reward, it integrates reinforcement, associative learning, and internal state into a single behavioral outcome (Cunningham *et al.*, 1997; McKendrick & Graziane, 2020; Flavell *et al.*, 2022). Positive reinforcement reflects the intrinsic rewarding value of a stimulus, whereas negative reinforcement reflects relief from discomfort or stress (Tzschentke, 2007; Navratilova *et al.*, 2013). In cocaine CPP, the experimental design is structured so that place preference predominantly reflects the reinforcing effects of cocaine, while aversive or stress-related influences are minimized (Tzschentke, 2007). In contrast, social CPP depends on both the intrinsic reward of peer contact and the negative reinforcement of avoiding isolation: without the final isolation phase, social CPP is not expressed (Panksepp & Lahvis, 2007). Social CPP therefore inherently captures stress-dependent and stress-independent motivational components, positioning the DYN/KOR system at the center.

Acute KOR activation suppresses dopamine release in corticolimbic circuits, generating dysphoria or aversion, while repeated or prolonged activation can induce long-lasting adaptations that sensitize reward pathways (Bruchas *et al.*, 2010; Knoll & Carlezon, 2010). Conversely, KOR antagonists, such

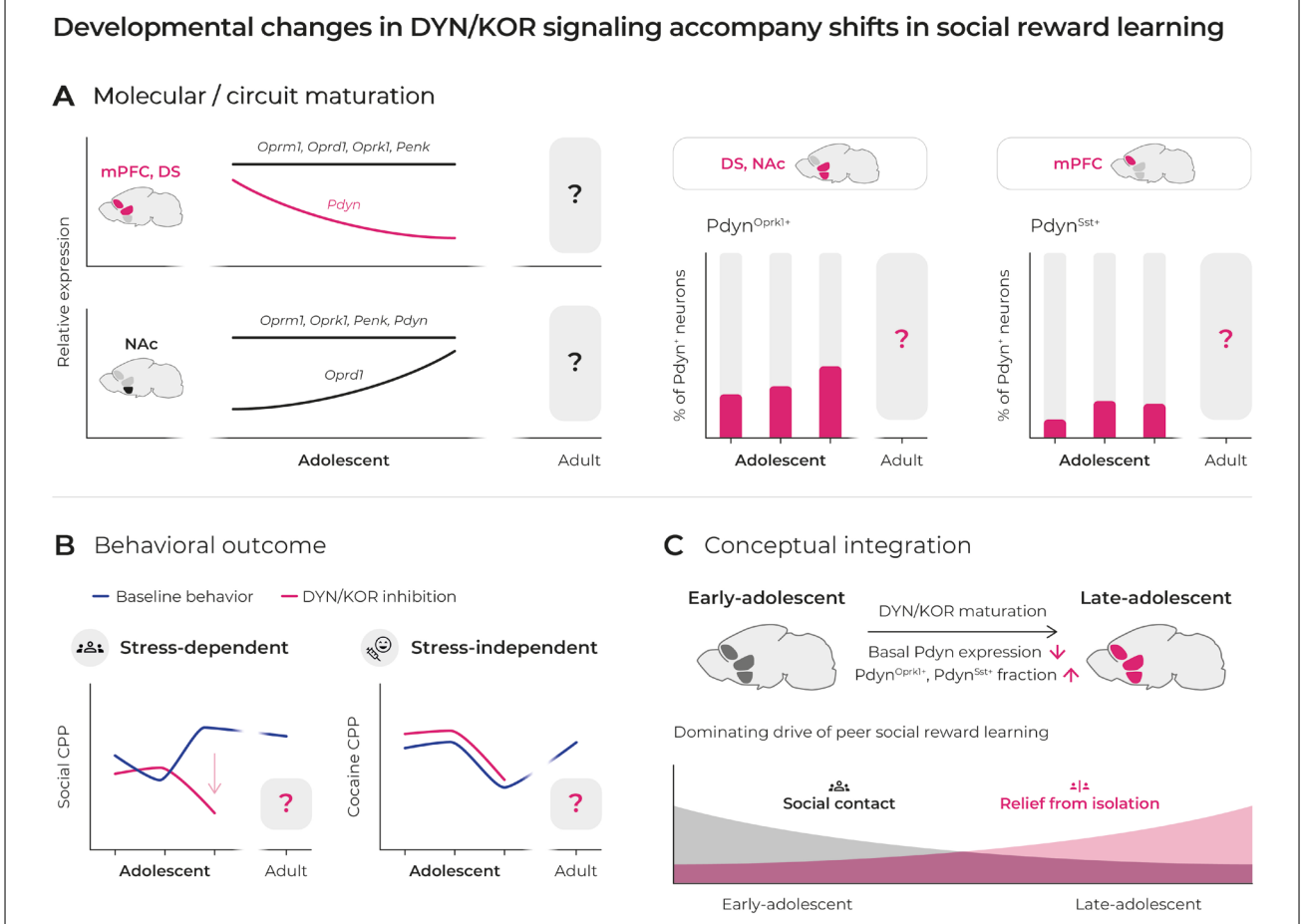

Figure 15. Behavioral and molecular shifts in adolescent DYN/KOR signaling identified in this PhD thesis

(A) Development of the endogenous opioid system. Curves show gene expression changes in the PFC (top) and striatum (bottom) for canonical opioid receptors (*Oprm1*, *Oprd1*, *Oprk1*) and peptide precursors (*Penk*, *Pdyn*). Pink curves indicate a developmental decrease in *Pdyn* expression in the mPFC and the DS. Co-localization of *Pdyn* and *Oprk1* is shown for the DS, the NAc (right), and the mPFC (far right). Question marks denote missing adult data.

(B) Influence of DYN/KOR inhibition on social and cocaine conditioned place preference (CPP) during adolescence. Blue curves depict baseline CPP acquisition; pink curves show CPP expression following KOR antagonism (norBNI) or *Pdyn* knockout. Light pink arrow indicates decreased CPP expression only in stress-dependent paradigm and in mature adolescents. Question marks denote missing adult data.

(C) Conceptual integration of findings. Maturation of the DYN/KOR system in the mPFC, the DS, and the NAc, characterized by decreased basal *Pdyn* expression and increased region-specific co-expression of *Pdyn and Sst* or *Oprk1*, may relate to developmental shifts in peer-social reward. These observations suggest that, as animals mature, social motivation could gradually shift from seeking intrinsic social contact toward relief from social isolation.

as norBNI, restore dopaminergic tone, preventing stress-induced inhibition and producing anxiolytic or antidepressant-like effects (Jackson *et al.*, 2013; Khan *et al.*, 2022). Under low-stress conditions, such as in the case of cocaine CPP, engagement of the DYN/KOR system is minimal. By contrast, social isolation robustly recruits stress-responsive networks involving the amygdala, bed nucleus of the stria terminalis, and NAc - regions densely populated by DYN-expressing neurons projecting onto KOR-expressing targets in the ventral striatum (Hang *et al.*, 2015; Limoges *et al.*, 2022). Activation of KORs within these circuits suppresses dopamine signaling, contributing to the dysphoric state characteristic of social isolation (Karkhanis *et al.*, 2016). This framework offers a plausible explanation for how our CPP paradigms distinguish intrinsic reward from stress relief. Cocaine CPP predominantly reflects basic reward-driven learning under low-stress conditions, whereas social CPP reveals how stress and reward dynamically interact during development. This might explain why cocaine CPP remains stable across adolescence, while social CPP undergoes changes (**Figure 15B**).

To assess the role of DYN/KOR signaling in CPP, I have used norBNI to block KOR activity during conditioning (Section 6.2, **Figure 8&9**). Previous work from our group demonstrated that acute administration of the selective KOR antagonist norBNI immediately before the posttest does not alter adolescent CPP expression, confirming that KOR antagonists influence acquisition rather than expression of stress-induced behavior (Jackson *et al.*, 2013; Khan *et al.*, 2022; Harda *et al.*, 2025). Thus, once learning has occurred, blocking KORs does not reverse the established behavioral pattern, the stress-related modulation has already shaped the preference.

I found that KOR blockade affected only social, but not cocaine CPP (Section 6.2, **Figure 8&9**). Administration of norBNI decreased social CPP only in late-adolescents, while early-adolescent social CPP remained unaffected (Section 6.2, **Figure 8**). To directly test the role of DYN in this developmental shift, I examined social CPP in *Pdyn* knockout mice (Section 6.2, **Figure 12**). The results showed the same age-dependent effect as norBNI administration: only late-adolescents exhibited reduced social CPP when DYN signaling was disrupted (Section 6.2,). In younger adolescents, DYN/KOR inhibition, either with norBNI or *Pdyn* knockout, did not reduce social CPP. At the same age, wild-type animals showed high basal *Pdyn* levels (Section 6.2, **Figure 2**). In older adolescents, DYN/KOR inhibition

decreased social CPP, which coincided with lower basal *Pdyn* in wild-types. These results reveal an age-dependent inverse relationship between basal *Pdyn* expression and DYN-modulated social reward.

These behavioral results collectively reveal two major developmental transitions in reward processing. The first involves a change in the nature of social motivation (**Figure 15B&C**). In early adolescence, direct peer interaction is enough to drive social CPP, as blocking KORs - which prevents isolation-induced stress - does not impair social reward learning. By late adolescence, however, direct social contact alone no longer drives CPP; preference increasingly depends on relief from isolation-induced stress, which requires mature DYN/KOR signaling. In other words, early adolescents may rely mainly on the intrinsic motivational value of social contact, whereas late adolescents may depend more on mechanisms that relieve social stress. This pattern aligns with typical developmental trajectories observed in rodents (Panksepp & Lahvis, 2007; Nardou *et al.*, 2019; Lin & Wilbrecht, 2022) and humans (Spear, 2000; Doremus-Fitzwater *et al.*, 2010; Galván, 2013; Spear, 2013): where social motivation toward peers tends to peak in early adolescence and then decline, whereas the drive to avoid isolation persists. Thus, these results indicate that in early life, social play may be the main driver of peer interaction, while with age, sexual and parental interactions may increasingly take precedence, diminishing the role of peer contact.

A second shift may involve the mechanisms underlying social motivation (**Figure 15B&C**). Although late adolescents still show strong social CPP, it is unclear whether this preference reflects the inherent rewarding value of social interaction or the activation of stress-sensitive circuits caused by isolation during the task. Other evidence suggests that KOR signaling matures during adolescence and may contribute to the shift in mechanisms driving social motivation: in juveniles, KOR activation can support prosocial or anxiolytic effects, whereas in adults it tends to produce dysphoria (Privette & Terrian, 1995; Braida *et al.*, 2009; Anderson *et al.*, 2014; Przybysz *et al.*, 2020; Spodnick *et al.*, 2025). Consistent with developmental transitions in KOR function, I found that inhibiting DYN/KOR signaling during conditioning reduces social CPP in older adolescents, indicating that stress-modulated reinforcement gains prominence as KOR signaling matures. Together, these findings support the idea of a

developmental switch in KOR function: initially buffering stress, then enhancing aversive responses and isolation-driven social motivation.

This raises a broader question: does the increasing role of stress-mediated mechanisms apply only to social rewards, or does it extend to other types of reward? While I did not directly test it, the cocaine CPP design minimized stress and likely limited KOR involvement; accordingly, pre-conditioning norBNI administration did not alter CPP magnitude at either age (**Figure 15B)**. Consistent with this, Montagud-Romero and collaborators found that acute social defeat stress enhanced cocaine CPP only in young adults (P50-53) but not in early adolescents (P29-32), ages comparable to those used in my thesis (Montagud-Romero *et al.*, 2015). This suggests that stress-potentiation of reward depends more on age and experimental context than on reward type and requires a mature DYN/KOR system. While DYN/KOR signaling may drive stress-dependent social CPP, it represents only one part of the opioid system's contribution to social reward. MOR/DOR signaling, central to the BOTSA (Panksepp *et al.*, 1980; Inagaki, 2018; Meier *et al.*, 2021), mediate the hedonic and affiliative aspects of social behavior. BOTSA posits that endogenous opioids enhance social pleasure and reduce separation distress, especially in juveniles. Supporting this, MOR agonists increase social play in juveniles, whereas MOR antagonists reduce it (Panksepp *et al.*, 1979; Vanderschuren, Niesink, *et al.*, 1995; Vanderschuren, Spruijt, *et al.*, 1995; Trezza *et al.*, 2011). In contrast, MOR agonists have limited effects on general social contact in adolescents and adults. Our previous work showed that acute norBNI before the posttest does not affect adolescent CPP expression, whereas acute antagonism of MOR or DOR enhanced social CPP in early- but not late-adolescents, indicating these mechanisms dominate in early adolescence and decline with age (Harda *et al.*, 2025). MOR/DOR action appears to modulate affective state, as administration of these antagonists alone does not produce CPP or CPA (Sikora *et al.*, 2019). This aligns with human studies showing that blocking opioid receptors with naltrexone modestly reduces feelings of social connection, suggesting that endogenous MOR signaling supports social bonding without being essential (Løseth *et al.*, 2024). Together, these findings suggest that developmental shifts in stress-mediated reward could influence not only social but also other types of rewards, and may be accompanied by opioid system maturation involving both KOR and MOR/DOR signaling.

In summary, adolescence is characterized by region- and cell-specific remodeling of *Pdyn* expression (**Figure 15A**). Early adolescents exhibit higher basal *Pdyn* in the mPFC and DS, with lower *Pdyn/Oprk1* co-localization in the DS and NAc and lower *Pdyn/Sst* co-localization in the mPFC relative to late adolescents. Across adolescence, basal *Pdyn* declines, potentially allowing for more context-dependent KOR recruitment. This maturation coincides with the emergence of stress-sensitive social CPP in late adolescents, whereas MOR/DOR signaling appears to support the intrinsic hedonic value of social contact, particularly in early adolescence (**Figure 15B**). These observations are consistent with the idea that developmental changes in *Pdyn* expression may contribute to shifts in social motivation across adolescence, with DYN/KOR signaling representing a key substrate for age-dependent engagement of stress-sensitive reward processes (**Figure 15C**).

7.4 Limitations and future directions

My study has significant limitations that constrain interpretation and urge caution when drawing conclusions. First, all molecular analyses and pharmacological manipulations were performed exclusively in adolescent male mice. Thus, whether the same changes occur in females and if they persist in adulthood is speculative. Second is causality. While I observed differences in basal *Pdyn* abundance and its co-expression with *Oprk1* or *Sst* between early- and late-adolescent mice, the functional consequences of these changes remain preliminary. The presence of a transcript does not guarantee the presence of protein, and even the presence of protein does not necessarily indicate functional activity. Additionally, my molecular analyses were limited to the mPFC, the DS, and the NAc, leaving out other key regions such as the amygdala, hippocampus, or ventral pallidum. These areas are critical for integrating stress and reward signals, and their exclusion limits my ability to fully understand how KOR-mediated circuits interact across the broader network that drives social and stress-related behaviors. The CPP paradigm imposes important constraints. Isolation stress is an inherent part of social CPP, whereas cocaine CPP was conducted under low-stress conditions. None of my paradigms was explicitly designed to quantify the influence of stress, limiting the ability to disentangle reward effects from stress-related motivational components.

These limitations set directions for future investigation. Including female subjects and studying adults would reveal whether the DYN/KOR changes are specific to males, to adolescence, or rather uniform across sexes and ages. Future studies could incorporate CPP paradigms with direct stress manipulations, such as prolonged early-life isolation, and examine social CPP alongside opioid gene expression in adulthood. Additional adolescent CPP paradigms, such as social CPP without prior isolation or social and cocaine CPP following experimentally induced stress, combined with genetic or pharmacological manipulation of DYN/KOR signaling, would help determine how this system regulates stress-dependent social motivation. These experiments could clarify whether age-specific effects of DYN/KOR inhibition on social CPP reflect a blockade of stress-potentiated social reward and whether this pattern extends to other reward types. Although separating the aversive effects of isolation from the reinforcing effects of peer interaction in the social CPP paradigm is challenging, studies that include MOR antagonists during social CPP acquisition could test whether the developmental shift in peer-directed social motivation, from predominant interaction reward to alleviation of isolation, truly exists. Real-time monitoring of DYN release, in vivo calcium imaging, and electrophysiological recordings, combined with optogenetic or chemogenetic targeting of *Pdyn*-positive neurons in the mPFC, NAc, and DS during CPP, would provide direct evidence of how DYN/KOR signaling modulates circuit activity. Extending these analyses to additional brain regions, such as the amygdala or hippocampus, could reveal how dynorphin-mediated changes shape coordinated network adaptations supporting flexible social behavior across development. Finally, selective rescue experiments, such as reinstating KOR signaling in *Pdyn* knockout mice, could test whether social CPP is restored when KOR function is normalized, providing causal evidence that maturation of the DYN/KOR system contributes to the developmental shift in social motivation.

Understanding how DYN/KOR signaling shapes social motivation across development has implications beyond basic neuroscience. Examining how stress and reward processes reorganize during adolescence can reveal the biological basis of social connectedness and vulnerability to stress-related disorders, helping explain why this period combines heightened social engagement with increased risk for affective or addictive behaviors. These studies may ultimately guide strategies to support adaptive social functioning and translate molecular insights into interventions that promote lifelong mental health.

7.5 Conclusions

Baseline reward sensitivity is lower during adolescence: Adolescent mice display reduced reward-conditioned place preference compared to adults, challenging the notion that adolescence is universally characterized by heightened reward sensitivity. This reduction does not appear to reflect deficits in learning, increased novelty-seeking, or altered locomotor activity. Instead, reward sensitivity is likely lower in low-salience contexts while remaining higher for socially or emotionally salient stimuli. Within adolescence, social and food CPP show stage-specific qualitative shifts, whereas cocaine CPP remains largely stable, suggesting that natural, but not drug, rewards undergo selective developmental changes.

Opioid system matures during adolescence: In adolescent male mice, *Pdyn* expression shows region- and cell type-specific changes: basal *Pdyn* expression decreases in the mPFC and the DS, *Pdyn/Oprk1* co-expression increases in the DS and NAc, and the proportion of $Pdyn^{Sst+}$ interneurons rises in the mPFC. These patterns suggest a developmental shift toward more context-specific and flexible regulation of social and stress-related behaviors.

Social reward transitions during adolescence: Social reward in non-reproductive contexts appears to change across adolescence. By late adolescence, social interactions may be valued more for relieving isolation-induced stress than for their immediate rewarding effects. This shift may reflect the gradual maturation of the DYN/KOR system, which increasingly enables stress to modulate reward processing. Overall, these findings suggest that adolescence represents a period of heightened plasticity in social and reward circuits, during which developing neural systems remain highly sensitive to social and stress-related cues that can shape adaptive or maladaptive social behaviors in adulthood.

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9. Declarations of co-authors

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Kraków, 22.10.2025 r.

OŚWIADCZENIE

Oświadczam, że udział mgr Klaudii Misiołek w niżej wymienionych publikacjach polegał na stworzeniu ogólnej koncepcji badań, wykonaniu części doświadczalnej, analizie wyników oraz przygotowaniu manuskryptów:

1. Misiołek K, Klimczak M, Chrószcz M, Szumiec Ł, Bryksa A, Przyborowicz K, **Rodriguez Parkitna J**, Harda Z. Prosocial behavior, social reward and affective state discrimination in adult male and female mice. Sci Rep. 2023 Apr 5;13(1):5583. doi: 10.1038/s41598-023-32682-6.

Mój udział w pracy polegał na:

Stworzeniu ogólnej koncepcji badań oraz udział w przygotowaniu manuskryptów

2. Misiołek K, Chrószcz M, Klimczak M, Rzeszut A, Netczuk J, Ziółkowska B, Szumiec Ł, Kaczmarczyk-Jarosz M, Harda Z, & **Rodriguez Parkitna J** (2025) *Adolescent mice exhibit lower reward sensitivity than adults.* Front. Behav. Neurosci., 19. doi: 10.3389/fnbeh.2025.1695375

Mój udział w pracy polegał na:

Stworzeniu ogólnej koncepcji badań oraz udział w przygotowaniu manuskryptów

Wyrażam zgodę na wykorzystanie niniejszej publikacji w postępowaniu doktorskim mgr Klaudii Misiołek oraz oświadczam, że wyniki nie zostaną ponownie wykorzystane w innych postępowaniach o nadanie stopnia doktora lub doktora habilitowanego.

.....

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Mój udział w pracy polegał na:

Stworzeniu ogólnej koncepcji badań (wspólnie z Klaudią Misiołek i Janem Rodriguezem Parkitną), przeprowadzeniu części doświadczeń, analizie części wyników i przygotowaniu pierwszej wersji manuskryptu.

2. Misiołek K, Chrószcz M, Klimczak M, Rzeszut A, Netczuk J, Ziółkowska B, Szumiec Ł, Kaczmarczyk-Jarosz M, **Harda Z**, & Rodriguez Parkitna J (2025) *Adolescent mice exhibit lower reward sensitivity than adults.* Front. Behav. Neurosci., 19. doi: 10.3389/fnbeh.2025.1695375

Mój udział w pracy polegał na:

Stworzeniu ogólnej koncepcji badań (wspólnie z Klaudią Misiołek, Janem Rodriguezem Parkitną i Barbarą Ziółkowską), asystowaniu przy niewielkiej części doświadczeń oraz pomocy w redakcji manuskryptu.

Wyrażam zgodę na wykorzystanie niniejszej publikacji w postępowaniu doktorskim mgr Klaudii Misiołek oraz oświadczam, że wyniki nie zostaną ponownie wykorzystane w innych postępowaniach o nadanie stopnia doktora lub doktora habilitowanego.

.....Zofia Harda.....

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Mój udział w pracy polegał na:

Pomocy w wykonaniu doświadczeń behawioralnych i przygotowaniu manuskryptu.

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Mój udział w pracy polegał na:
wykonaniu doświadczeń behawioralnych, edycji manuskryptu

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Magdalena Chrószcz

Mgr Magdalena Chrószcz

Instytut Farmakologii
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Polskiej Akademii Nauk

Zakład Neurofarmakologii Molekularnej

Kraków, 22.10.2025 r.

OŚWIADCZENIE

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1. Misiołek K, Chrószcz M, Klimczak M, Rzeszut A, Netczuk J, **Ziółkowska B**, Szumiec Ł, Kaczmarczyk-Jarosz M, Harda Z, & Rodriguez Parkitna J (2025) *Adolescent mice exhibit lower reward sensitivity than adults.* Front. Behav. Neurosci., 19. doi: 10.3389/fnbeh.2025.1695375

Mój udział w pracy polegał na uczestnictwie w planowaniu badań, interpretacji wyników i dopracowaniu manuskryptu.

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B. Ziółkowska

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Dr hab. Barbara Ziółkowska

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im. Jerzego Maja
Polskiej Akademii Nauk

Zakład Neurofarmakologii Molekularnej

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Aleksandra Rzeszut

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Zakład Neurofarmakologii Molekularnej

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Dr inż. Maria Kaczmarczyk-Jarosz

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Zakład Neurofarmakologii Molekularnej

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Mój udział w pracy polegał na analizie danych pochodzących z testów behawioralnych rozpoznawania stanu afektywnego przeprowadzonych na myszach.

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Mgr Karolina Przyborowicz

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Zakład Neurofarmakologii Molekularnej

Kraków, 04.11.2025 r.

OŚWIADCZENIE

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Mój udział w pracy polegał na:
uczestnictwie w części behawioralnej pracy.

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Julia Netczuk

Mgr Julia Netczuk

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NETCZUK
Data/ Date: 04.11.2025 14:33
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10. Scientific articles in their original version

10.1 Original paper 1

Misiołek K, Klimczak M, Chrószcz M, Szumiec Ł, Bryksa A, Przyborowicz K, Rodriguez Parkitna J, (2023) Harda Z. *Prosocial behavior, social reward and affective state discrimination in adult male and female mice.* Sci Rep. doi: 10.1038/s41598-023-32682-6

10.2 Original paper 2

Misiołek K, Chrószcz M, Klimczak M, Rzeszut A, Netczuk J, Ziółkowska B, Szumiec Ł, Kaczmarczyk-Jarosz M, Harda Z, & Rodriguez Parkitna J (2025) *Adolescent mice exhibit lower reward sensitivity than adults.* Front. Behav. Neurosci., 19. doi: 10.3389/fnbeh.2025.1695375

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Prosocial behavior, social reward and affective state discrimination in adult male and female mice

Klaudia Misiołek[1], Marta Klimczak[1], Magdalena Chrószcz[1], Łukasz Szumiec[1], Anna Bryksa[1,2], Karolina Przyborowicz[1], Jan Rodriguez Parkitna[1]✉ & Zofia Harda[1]✉

Prosocial behavior, defined as voluntary behavior intended to benefit another, has long been regarded as a primarily human characteristic. In recent years, it was reported that laboratory animals also favor prosocial choices in various experimental paradigms, thus demonstrating that prosocial behaviors are evolutionarily conserved. Here, we investigated prosocial choices in adult male and female C57BL/6 laboratory mice in a task where a subject mouse was equally rewarded for entering any of the two compartments of the experimental cage, but only entering of the compartment designated as "prosocial" rewarded an interaction partner. In parallel we have also assessed two traits that are regarded as closely related to prosociality: sensitivity to social reward and the ability to recognize the affective state of another individual. We found that female, but not male, mice increased frequency of prosocial choices from pretest to test. However, both sexes showed similar rewarding effects of social contact in the conditioned place preference test, and similarly, there was no effect of sex on affective state discrimination measured as the preference for interaction with a hungry or relieved mouse over a neutral animal. These observations bring interesting parallels to differences between sexes observed in humans, and are in line with reported higher propensity for prosocial behavior in human females, but differ with regard to sensitivity to social stimuli in males.

Prosocial behavior, defined as acting willfully to meet the perceived need of another individual, is regarded as the highest form of empathy[1,2]. In humans, a major factor affecting the propensity for prosocial behaviors is gender[3]. Accumulated evidence indicates that females have superior emotion discrimination abilities[4], are more concerned about the well-being of others[5], and utilize more resources to support others in need[6]. Some previous reports have argued that altruistic, prosocial behavior is a uniquely human characteristic (e.g.,[7,8]); however, a growing number of reports show that targeted helping is also observed in other species. Laboratory rodents (rats:[9–11], mice:[12,13], but see[14]) and some bird species[15] prefer actions that reward another conspecific in choice tasks or free another animal from a restraint, with whom they share a food reward afterward. Unlike in humans, there are limited data on the effect of sex on prosocial behaviors in laboratory animals. Most rodent studies on affective state discrimination have focused on only one sex, usually males (for reviews, see rats and mice:[16,17]), although some studies have investigated females (e.g., rats:[18–20]). Several studies examined both females and males, but the results considering sex-differences appear inconclusive (rats and mice:[21], rats:[9,22,23] mice:[24–26]). Some of the reports suggest that females are more susceptible to emotional contagion (mice:[25]), show enhanced emotion discrimination abilities in double approach paradigms (mice:[24], rats:[27]) are more likely to perform prosocial actions (rats:[9,28]). However, other studies provide evidence for equal susceptibility of male and female rodents to emotional contagion (rat and mice:[21], mice:[22,29–31]), equal affective state discrimination skills (mice:[26]), and comparable levels of prosocial behaviors (rats and mice:[21], rats:[32], prairie voles:[33]). Interestingly, some authors observed even higher levels of empathy-motivated behaviors in male rodents (rats and mice:[21], rats:[23,34], mice:[12,35]). Thus, previous reports appear inconsistent with regard to superior empathic and prosocial abilities in female mice.

Recently, it was proposed that prosocial behavior is promoted by the positive affective state of the recipient and witnesses of the interaction, and is motivated by the rewarding effects of social interactions and empathy termed together as the "camaraderie effect"[36]. The "camaraderie effect" theory offers a plausible explanation for the reported differences in prosocial behaviors in mice[12–14,35], pointing at the differences in levels of social reward and empathy under different experimental setups. Moreover, based on the "camaraderie effect" we hypothesized

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that sex differences in prosocial behavior should be reflected in the rewarding effects of social interactions and affective state discrimination. Thus, we first investigated prosocial choices in adult male and female C57BL/6 laboratory mice, towards a familiar partner (sibling). We used a model based on the general outline of the rat task described by Hernandez-Lallement and collaborators[10]. In our task, mice were provided with a choice between delivering food to themselves or to both themselves and a partner. Social reward was tested in the social conditioned place preference task[37] (sCPP) and affective state discrimination was tested in a paradigm modified from Scheggia et al. in 2020, where sensitivity to the emotional state of interaction partners was assayed[26]. We found that, similar to humans, sex had a significant effect on the propensity for prosocial choices in mice. However, female and male mice were not different in sensitivity to social reward or the affective state of a conspecific.

Results

Prosocial choices in adult male and female mice. To assess prosocial choices in adult mice, we used a custom-made maze, as shown in Fig. 1a. In the task, a focal animal, the actor, chose to enter one of two compartments and would be rewarded with chocolate chips for either choice. A second animal, the partner, also received a reward, but only if the actor entered the compartment designated "prosocial". The wall separating the actor's and partner's compartments was transparent and perforated, allowing for visual, auditory and olfactory communication. The schedule of the experiment is summarized in Fig. 1b. First, the actor animals underwent up to 4 pretest sessions, one session per day, without partner animal (Fig. 1c). The number of chocolate chips consumed was checked after each trial, and only animals that consumed at least 85% of the chips over two consecutive days were subjected to the actual test (Fig. 1d). The average number of sessions required to reach this criterion was 2.4 and 2.63 in male (n = 10) and female (n = 8) mice, respectively (t-test, $t_{16} = 0.76$, $p = 0.46$, Fig. S1a–d, Table S1).

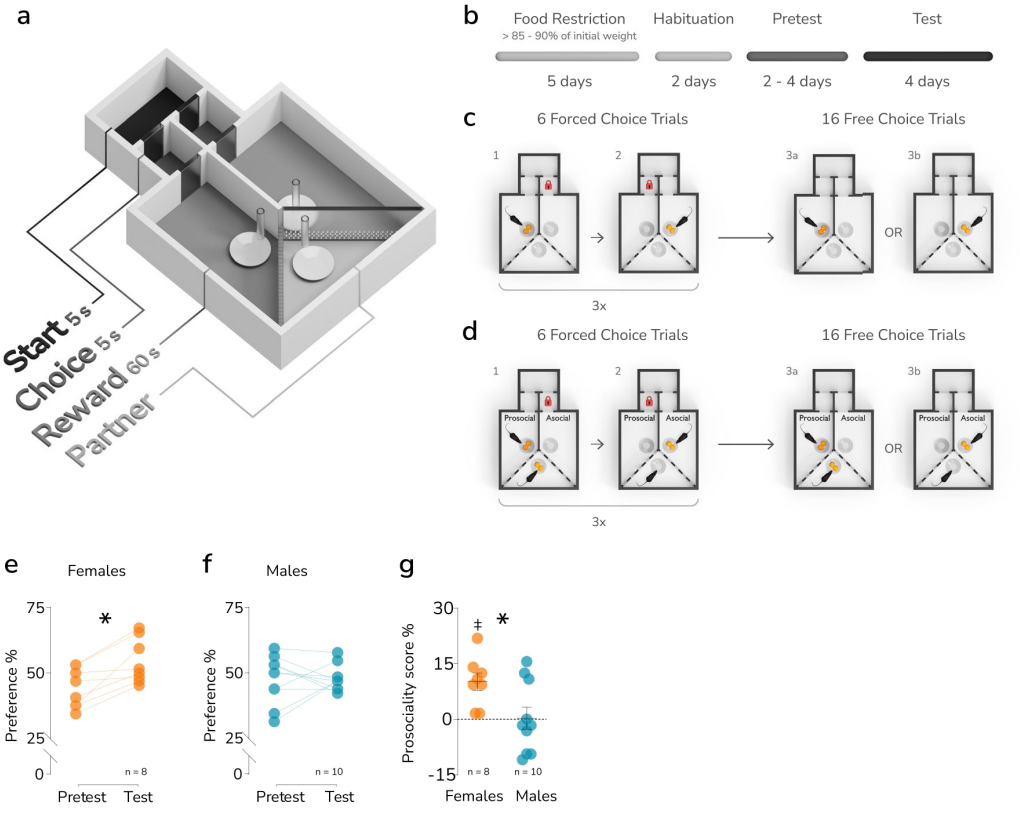

Figure 1. Prosocial choices in male and female mice. (**a**) A schematic representation of the testing apparatus. (**b**) Experimental schedule. (**C–D**) A diagram summarizing the pretest and test phases of the experiment, respectively. (**e–f**) The change in preference for the 'prosocial' compartment between the pretest and test phases in female and male mice, respectively. The results shown are the mean values from all sessions in the pretest and test phases. A significant difference between the phases is marked with a "*" (paired t-test $p < 0.05$). Respective group sizes are indicated below the graphs. (**g**) The difference between females and males prosociality score, calculated as test preference for the prosocial compartment minus pretest preference for the same compartment (%). The bar and whiskers represent the mean and s.e.m. values. A significant difference between means is marked with a "*" (t-test $p < 0.05$), and a significant difference vs. 0 is indicated by a "‡" (one-sample t test $p < 0.05$). The group sizes are indicated below the graph.

The pretest was intended to train actors and to assess their inherent preference between the left and right compartments in the absence of a partner animal. No inherent preference for cage side was detected in males or females (Fig. S1e, Table S2).

Then, the partners were introduced, and one of the compartments was designated "prosocial" (Fig. 1d). Four test sessions were performed. The frequency of entries to the prosocial compartment during the test was compared to the frequency of entries to the same compartment ("prosocial to be") during the pretest. In female mice, the preference for the prosocial compartment increased significantly (Fig. 1e, Table S2), while male animals appeared to show no change from their initial choices (Fig. 1f, Table S2). In the case of females, the preference for prosocial behavior changed from 44.14% initially to 54.30% (average from the 4 trials, paired t-test, $t_7 = 4.33$, $p = 0.003$), while in males, these values were 47.52% and 47.83%, respectively. The difference in the prosociality score (defined as the percentage of prosocial choices during the test minus the percentage of prosocial choices during the pretest) between males and females was significant (Fig. 1g, Table S3, t test, $t_{16} = 2.47$, $p = 0.025$). Additionally, we examined correlations between the absolute and relative weights of the actor and partner and the prosociality score. We have assumed that heavier mice were more likely to be dominant, and thus the weight difference was a proxy of the social hierarchy. Relative weight was defined as the difference in weight between actor and partner calculated as a percentage of actor's weight. The analysis revealed no significant association for absolute weights in any of the sexes and no significant correlation of relative weight in the case of females (Table S4). However, a negative correlation between relative weight and prosociality score was observed in male mice (Table S4, $r = -0.73$, $p = 0.014$). These results could indicate that in the case of males the greater the weight difference between partner and actor, the fewer prosocial choices were made. Together, these results show that female mice favored prosocial choices in the task, while males have shown no preference for either choice.

Social reward. An increase in the frequency of prosocial choices observed in females is evidence of reinforcing effects of their consequences and, thus, of a rewarding effect of the choice. No preference for the prosocial choices in male mice could potentially be attributed to a generally lower sensitivity to the rewarding effects of social interaction. To assess this possibility, we tested a separate cohort of adult mice of both sexes in the sCPP, with a 6 days conditioning protocol[37]. In this test, experience of social contact during conditioning causes an increase in time spent in the previously neutral context from pretest to posttest. Both female (n = 16) and male (n = 12) mice significantly increased the time spent in the context associated with group housing (Fig. 2a, paired t-test, $t_{15} = 2.825$, $p = 0.012$, Fig. 2b, t-test, $t_{11} = 4.202$, $p = 0.002$, Table S5). Likewise, the preference score (i.e., the difference in time spent in the social minus isolate context during the posttest) was significantly higher than chance value in both female (one sample t-test, $t_{15} = 3.282$, $p = 0.005$) and male (t-test, $t_{11} = 2.446$, $p = 0.033$) mice (Fig. 2c, Table S6). Moreover, there was no difference between males and females in preference score (t-test, $t_{26} = 0.5$, $p = 0.62$). These results indicate that social interactions with siblings were rewarding for male and female mice to similar extent. The finding that sCPP can be elicited in adult mice using contact with age and sex matched conspecifics as a reward appears contradictory to the previous findings by Nardou and collaborators in 2019, who have shown that sCPP was no longer observed at the age of 8 weeks in male, and at 11 weeks in female mice[38]. However, in the study the short (2 days) conditioning protocol was used[38], and we have recently shown that sCPP can be observed in adult (> 11 weeks) female mice, when the longer, 6 days, conditioning protocol is used[37]. Here, to assess if the effect of conditioning length is similar in males, we performed additional experiment on male mice (n = 8) using a 2 day conditioning protocol (Fig. 2d–e, Tables S5–S6). We found no effect of social conditioning, which is in agreement with the results reported in 2019 by Nardou and collaborators[38].

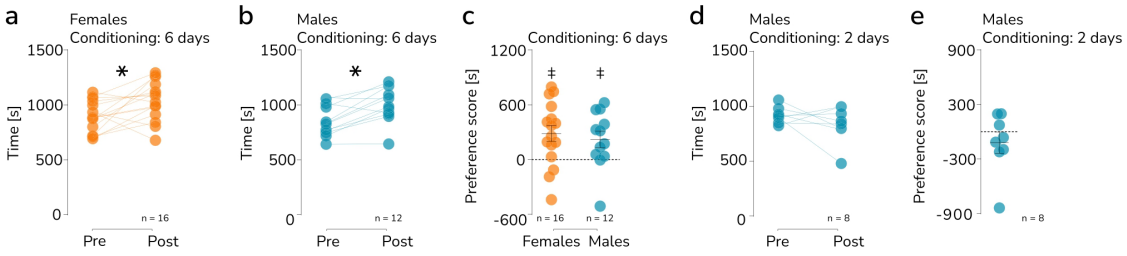

Figure 2. Social conditioned place preference. (**a–b**) The change in preference for the social context after 6 days of conditioning in female and male mice, respectively. Each pair of points represents an individual animal, and the group sizes are indicated below the graphs. A significant difference between the pre- and posttests (30 min) is marked with a "*" (paired t-test, $p < 0.05$). (**c**) No difference between females and males preference score [s], calculated as time spent in social context posttest [s] minus time spent in in isolate context posttest [s]. Each point represents an individual female or male mouse, with the mean and s.e.m. shown in black and the group sizes indicated below. A significant preference (greater than 0) is indicated by a "‡" (one-sample t-test $p < 0.05$). (**d**) No significant change in preference for the social context after 2 days of conditioning in male mice. (**e**) The preference score for social context during the posttest after 2 days of conditioning. The group sizes are indicated below the graph.

Sensitivity to the affective state of the interaction partner. A plausible explanation for the observed effect of sex on prosocial choices would be a difference in sensitivity to the affective state of the interaction partner. To test this possibility, a separate cohort of mice was assayed for their preference for interaction with a "neutral" vs. altered affective state demonstrator (positive-"relieved" or negative-"hungry") in a affective state discrimination procedure based on the task described in 2020 by Scheggia et al.[26] and summarized in Fig. 3a. During the main phase of the test, the animal tested (observer) was placed in the cage where demonstrators were present, both confined under wire cups. One of the demonstrators was food deprived: for 24 h (hungry) or for 23 h and then offered ad libitum food access (standard laboratory chow) for 1 h preceding the test (relieved). The second demonstrator (neutral) as well as the observer had constant ad libitum food access. Both female (n = 12) and male (n = 13) observer mice spent significantly more time sniffing the relieved demonstrator during the first minute of the test (Fig. 3b–c, Table S7; Fig. 3b, repeated measures ANOVA, effect of time, $F_{(2.94, 64.62)} = 3.69$, $p = 0.017$, Šídák's test, sniffing relieved vs. neutral demonstrator during first minute, $p = 0.019$; Fig. 3c, effect of demonstrator's state, $F_{(1, 24)} = 6.233$, $p = 0.012$, Šídák's test, sniffing relieved vs. neutral demonstrator during first minute, $p = 0.02$), and there was no effect of sex on the fraction of time spent sniffing the relieved demonstrator (Fig. 3d, Tables S8–S9). The preference for relieved demonstrator was most evident during the first minute of the observation, which corroborates the results obtained earlier[26]. Thus, only the behavior during this time period was considered an indication of affective state discrimination. Similarly, both female (n = 8) and male (n = 10) observer mice spent significantly more time sniffing the hungry demonstrator during the first minute of the test (Fig. 3e-f, Table S7; Fig. 3e, repeated measures ANOVA, effect of interaction of time × demonstrator's state, $F_{(3, 42)} = 5.157$, $p = 0.004$, Šídák's test, sniffing hungry vs. neutral demonstrator during first minute, $p = 0.008$; Fig. 3f, effect of interaction of time × demonstrator's state, $F_{(3, 54)} = 5.391$, $p = 0{,}003$, Šídák's test, sniffing hungry versus neutral demonstrator during first minute, $p = 0.026$), and there was also no effect of sex (Fig. 3g, Tables S8–S9). The position of the altered affective state demonstrator (east vs. west side of the testing apparatus) was selected randomly, and no effect of the relieved or hungry demonstrator's position on the percentage of time spent sniffing this demonstrator was found (Figs. S2a–b, S2f–g, Table S10). Additionally, to control for inherent preference of the position of the cups (east vs. west), we also analyzed the time spent sniffing the cup in which the relieved/hungry demonstrator would be placed during the last day of adaptation. The time spent sniffing the empty cup did not differ from chance level (Figs. S2e, S2h, Table S11) except for females paired later with relieved demonstrators. In this case time spent sniffing the empty cup was significantly shorter than the chance level (Fig. S2e, t-test, $t_8 = 2.495$, $p = 0.037$). Finally, we also analyzed the correlations between the fraction of time spent sniffing the relieved/hungry demonstrator and the weight of the animals, both observers and demonstrators (as well as the weight difference between them as an indicator of their hierarchy). This analysis revealed a significant negative correlation for both relieved demonstrator ($r = -0.7$, $p = 0.001$) and observer weight ($r = -0.62$, $p = 0.028$), as well as a trend towards observer-relieved demonstrator weight difference ($r = 0.53$, $p = 0.073$), in females (Table S4). These results could indicate greater preference of the partner in "relief" state among smaller females. No significant correlations were found in the case of hungry demonstrators (Table S4). In general, the correlations between weight differences and prosocial choices or affective stated discrimination do not indicate a consistent effect of hierarchy on the observed behaviors. Taken together, these results show that both female and male mice discriminate between affective states of familiar conspecifics. Thus, there is no evidence of an effect of sex on sensitivity to affective states of conspecifics.

Discussion

We found that female, but not male, C57BL/6 mice showed significant preference for prosocial behavior toward a familiar partner. Recently, Scheggia and colleagues showed that altruistic, prosocial behaviors in mice are dependent on sex, familiarity, social hierarchy, and also internal and affective state[12]. They observed that male mice were more likely to share food with same sex conspecific than females in the operant two-choice social decision-making task, which is at odds with our results. This discrepancy may emerge from differences in methodology. In the report cited, testing was performed in an automated operant chamber, whereas we have used a manually operated cage. This could have affected animal's stress levels and thus bias choices. Furthermore, Scheggia and collaborators found that tactile social contact is necessary for prosocial choice preference development in male mice[12]. When partition dividing actor and partner lacked perforations, the focal animals made fewer prosocial decisions. In our experiment transparent and perforated partition between actor's and partner's compartments was used, to allow access of visual, auditory and olfactory cues. However, due to the small size of the perforations, direct contact between mice was restricted, which speculatively could influence preference of prosocial choice in males. Our findings, together with the results reported by Scheggia and colleagues[12], suggest that lower level of tactile contact might decrease prosocial behavior in male, but increase in female mice. Indeed, there is evidence for an effect of sex on processing of tactile contact. Experiments performed on rats showed that regular-spiking neurons in the barrel cortex exhibited stronger responses to facial touch (nose-to-nose) in males compared to females[39]. Future studies should directly test the relationships between sex, social touch and prosocial behavior.

It should be noted that the observation that female mice are more prosocial than males in a food-motivated prosocial choice task is in accordance with other findings in rats using different measures of prosociality. In 2011 Ben-Ami Bartal and colleagues found that females are more likely to learn how to free a trapped cagemate, and, when the task is learned, females perform it faster than males[9]. Furthermore, in 2020 Heinla and colleagues found that female, but not male, rats show consolation-like behavior directed toward cagemates that were recently attacked by another individual[28].

Based on the "camaraderie effect" theory[36], we hypothesized that a female advantage in prosocial behavior may stem from higher emotion discrimination abilities or higher rewarding effects of female–female, compared with male–male, social interactions. However, no sex difference in these behaviors was observed. The finding that

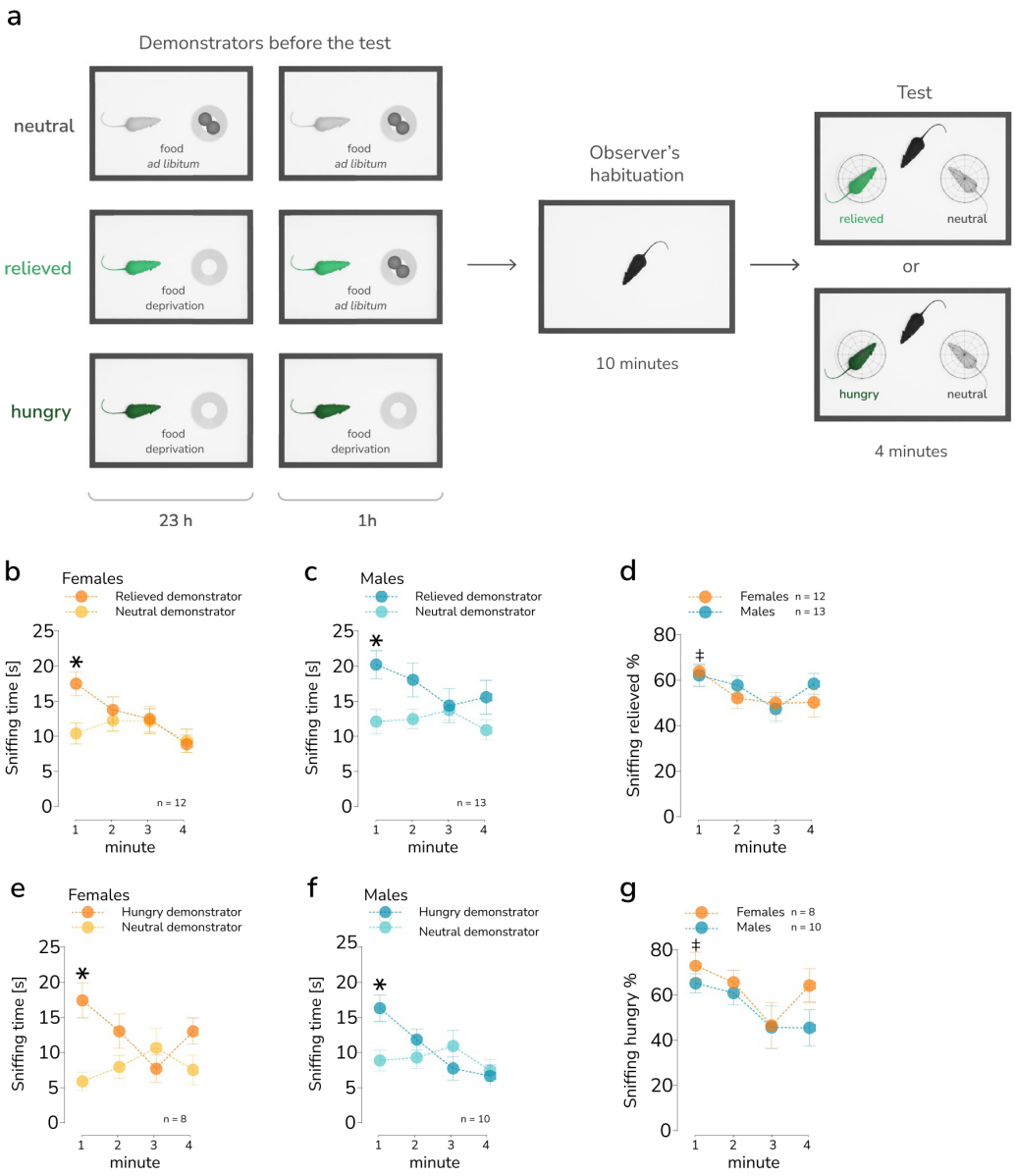

Figure 3. Affective state discrimination. (**a**) A schematic representation of the task. (**b–c**) The time spent by the demonstrator sniffing the relieved (darker points) and neutral (lighter points) demonstrators, female and male mice, respectively. Each point represents the mean time spent sniffing respective partners during a 1 min interval (bin). The whiskers represent s.e.m. values, and significant differences between the mean time spent sniffing in the first 1 min interval are shown with a "*" (ANOVA with repeated measures; post hoc Šídák's multiple comparisons test, $p < 0.05$). (**d**) No difference between female and male mice in preference for sniffing the relieved demonstrator over the neutral demonstrator. The points represent mean values over 1 min intervals, whiskers represent the s.e.m. Significant differences vs. 50% during the first 1 min interval in both female and male mice are indicated by a "‡" (one-sample t-test $p < 0.05$). (**e–f**) The time spent by the demonstrator sniffing the hungry (darker points) and neutral (lighter points) female and male demonstrators, respectively. Each point represents the mean time spent sniffing respective partners during a 1 min interval (bin). The whiskers represent s.e.m. values, and significant differences between the mean time spent sniffing in the first 1 min interval are shown with a "*" (ANOVA with repeated measures; post hoc Šídák's multiple comparisons test, $p < 0.05$). (**g**) No difference between female and male mice in preference for sniffing the hungry demonstrator over the neutral demonstrator. The points represent mean values over 1 min intervals, whiskers represent the s.e.m. Significant differences versus 50% during the first 1 min interval in both female and male mice are indicated by a "‡" (one-sample t-test $p < 0.05$). The group sizes are indicated below the graph.

emotion discrimination abilities, independently from valence, are similar in male and female mice corroborates previous observations Scheggia and colleagues[26], nevertheless, some of the previous reports showed higher emotion discrimination abilities in female rodents (mice:[24], rats:[27]). These discrepancies may stem from the differences in the severity of the emotion-eliciting stimulus and/or emotion valence. In our study, a positive state was induced by deprivation and subsequent provision of food and the negative state was induced by food deprivation for 24 h before the test, which could be considered as a relatively mild stress. Scheggia and collaborators used a test where a positive state was induced by deprivation and subsequent provision of water and the negative state was induced by 15 min of restraint before the test, which also is arguably a mild stressor. Conversely, studies that have demonstrated higher emotion discrimination abilities in female rodents used severe stimuli, i.e. pain or footshock (mice:[24], rats:[27]). A conjecture that sex differences in affective state discrimination are evident only in the test involving highly stressful stimuli would be in agreement with "fitness threat hypothesis", which states that female advantage in emotion recognition may be limited to negative emotional expressions, as they signal a potential threat to the offspring[40].

The finding that rewarding effects of social interactions are similar in adult male and female mice is especially surprising, as males of the *Mus musculus* species studied in natural or seminatural conditions usually have been found to form territories and aggressively defend them from other males[41]. Female mice, in contrast, are capable of communal nesting and nursing[42]. Both male and female mice disperse from their natal groups, but males do this more frequently and at younger ages[43]. Taken together, these literature data suggest that the motivation for the social context preference observed here may differ between males and females. In females, amicable social interactions are the most likely cause of the increase in the preference for social context. In males, however, the opportunity for aggressive encounters may have caused the same effects. Earlier studies support this interpretation, as rewarding effects of aggression were repeatedly documented in male mice and rats (for a review, see[44]), but were absent in female mice[45].

A potential limitation of our study is that in the affective state discrimination test the food deprivation was used instead of water deprivation, as previously described[26]. This leads to a question if observer mice indeed have shown preference for the emotionally aroused conspecific or rather the one that emitted more intense food odor. However, this issue is mostly resolved by the comparison of "relieved" and "hungry" conditions. If, in the "relieved" condition, relieved demonstrators emitted more smell of the food than neutral demonstrators, then, consequently, in the "hungry" condition, neutral demonstrators would have emitted more intense food odor than hungry demonstrators. If observer mice were attracted to the smell of food rather than the emotional arousal of the demonstrators, in the "hungry" condition they would have explored the neutral demonstrator more than a hungry one, which was not the case. Hence, we believe that in our version of the task mice indeed show recognition of emotional states of others, not interest in food odor.

Taken together, our results show that, similar to humans, female mice tend to be more prosocial than males, but this difference may not stem from sensitivity to affective state of the interaction partner or stronger rewarding effects of social interactions. Thus, the relationships among prosociality, affective state discrimination and social reward should be reconsidered, and correlations between these traits are not indicative of causation.

Materials and methods

Animals. Experiments were performed on C57BL/6 mice bred at the Maj Institute of Pharmacology Animal Facility. Mice were housed in a 12-h light–dark cycle (lights on at 7 AM CET/CEST) under the controlled conditions of 22 ± 2 °C temperature and 40–60% humidity. In the prosocial choice test, mice were housed as sibling pairs. For affective state discrimination and sCPP mice were housed with littermates of all the same sex or alone, depending on the phase of the experiment. Water was available ad libitum. Home cages contained nesting material and aspen gnawing blocks. Behavioral tests were conducted during the light phase under dim illumination (5–10 lx). Affective state discrimination and sCPP tests were video recorded with additional infrared LED illumination. The age and weight of all experimental groups are summarized in Table S12.

All behavioral procedures were approved by the II Local Bioethics Committee in Krakow (permit numbers 224/2016, 34/2019, 35/2019, 32/2021) and performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The reporting in the manuscript follows the ARRIVE guidelines.

Prosocial choice test. The method was partly based on the prosocial test for rats described in 2014 by Hernandez-Lallement and colleagues[10]. The custom cage (main compartment: 30 × 36 × 30 cm; start-choice compartment: 12 × 14 × 30 cm) used in the procedure is shown in Fig. 1a. The walls between reward and partner compartments were transparent and perforated so animals could see, hear, and smell each other during the experiment. Two pairs of doors were used for each arm of the apparatus to prevent the animal from going back to the starting compartment. Animals were given limited time to enter the choice compartment (5 s) and reward compartments (5 s). In case mice didn't make a choice in time, animals were gently touched by hand of the experimenter to encourage them to enter either of the reward compartments. Entering one of two reward compartments resulted in food delivery after 5 s. The time to consume the reward was also limited (60 s). Animals were moved to starting compartment if they consumed both rewards or 60 s had passed. Time between each trial lasted 10 s. The primary difference from the previously described apparatus is the single compartment for the interaction partner. The experimental schedule is summarized in Fig. 1b and consisted of 4 phases: food restriction (5–7 days), habituation (2 days), pretest (2–4 days, depending on completion criterion; see Table S1), and main test (4 days). Mice had restricted access to food throughout the experiment. Habituation was performed when animals reached 85–90% of their initial body weight. On the last day of food restriction, the heavier mouse from each cage was selected as the actor, and the lighter mouse was used as the partner. The rationale was to

increase the chance of observing prosocial behavior in actors, as it was shown that the number of reward portions provided to the hungry partner is negatively correlated with the partner's weight in rats[10,20] Habituation took place on the two days preceding the pretest. Actors and partners were placed in the assigned compartments for 10 min to freely explore all compartments. Reward was available ad libitum. During the pretest, only actors were present in the cage. The pretest session consisted of 6 forced choice trials and 16 free choice trials. The sequence of forced trials was always an alternation of right and left choices, starting with right.

At the beginning of each trial, the actor was placed in the starting compartment. Then, the doors were lifted, and the actor could access one of the reward compartments (during forced trials) or was offered a choice between the two compartments (during free choice trials). The actor received a food reward irrespective of choice (two chocolate chips, BioServ, 20 mg, #F05301). After the actor consumed the reward, it was placed back in the starting compartment. The time mice could spend in each of the compartments was limited (Fig. 1a). In case the mouse did not move to the desired compartment before the time limit, the experimenter gently pushed it. The completion criterion for the pretest was 37 out of 44 food pellets consumed in two consecutive sessions (for the number of animals excluded based on this criterion, see Fig. S1 and Table S1). Additionally, an exclusion criterion was a > 70% average preference for one of the compartments (the '70% criterion'). Number of animals that passed the predefined criteria is n = 8 for females and n = 10 for males.

During the main test phase, both the actor and partner were introduced to the cage, and testing sessions were performed daily over 4 days. Each actor's entry to the "prosocial" compartment resulted in reward delivery for both mice. Conversely, upon entrance to the "asocial" compartment, only the actor was rewarded. The prosocial compartment was assigned as follows: in the case of mice with less than a 60% preference, the compartment was selected randomly. For the mice with an initial preference between 60 and 70%, the less preferred compartment was chosen as prosocial.

We considered the possibility that the prosocial behavior in female mice could be affected by estrous cycle phase. The estrous cycle in mice lasts for approximately four days. To minimize the possible effect of estrous cycle phase on the differences between males and females, the 4 days average of the test phase results was used for pretest–posttest comparison and for comparison between sexes. The 4 days average was used for both sexes, to enable male–female comparison.

Social conditioned place preference (sCPP). sCPP was performed as previously described[37,46]. The procedure consisted of three phases: pretest, conditioning phase, and posttest.

During pretest each cage compartment contained one type of context (context A and context B) that differed in bedding type and gnawing block size and shape (Table S13). Both conditioning contexts were different from the home cage context, which consisted of aspen bedding (Table S13) and a distinct gnawing block. Mice were allowed to freely explore the cage for 30 min. Two animals were tested in parallel in adjacent cages. The exclusion criterion for pretest was initial preference to any of the context exceeding 70% (for the number of animals excluded based on this criterion, see Table S14). Number of animals that passed the predefined criteria is n = 16 for females, n = 12 for males tested in the 6 days protocol and n = 8 for males tested in 2 days protocol.

After the pretest, animals were returned to their home cages for approximately 24 h. Then, mice were subjected to social conditioning (housing with cage mates) for 24 h in one of the contexts used in the pretest followed by 24 h of isolated conditioning (single housing) in the other context. To preserve an unbiased design, the social context was randomly assigned in such a way that approximately half of the cages received social conditioning on context A and half on context B. The conditioning phase lasted 6 days (3 days in each context, alternating every 24 h). After conditioning, the post-test was performed identically as pretest. Two sets of conditioning contexts were used (Table S13), and the results from both sets were pooled. When the number of animals conditioned on different bedding types (contexts) were not equal, the number of animals for each type of bedding was equalized by randomly removing the appropriate number of cases from the larger group using an R script as described in[37].

Affective state discrimination. The test was based on the protocol developed in 2020 by Scheggia and colleagues[26]. The behavior was assessed in a rectangular cage with opaque walls (see Fig. 3A; 53 cm × 32 cm × 15 cm). Demonstrators were placed on plastic platforms and confined under wire cups (diameter 9.5 cm × height 9 cm, Warmet, #B-0197). The procedure comprised two phases: habituation (3 days) and testing (1 day). The largest animal in the cage at the start of habituation was always assigned the "observer" role, the second largest was assigned the "relieved" or "hungry" demonstrator role, and the smallest was assigned the "neutral" demonstrator. This was done to match the role assignment in the prosocial choice task and to ensure that the relieved/hungry and neutral demonstrators had a similar weight such that the only characteristic that distinguished them was the affective state. The relieved/hungry demonstrator and observer were always naïve, while the neutral demonstrators were tested twice with different observers in 9 cases, always a week apart.

On the first day of habituation, the observers were placed in the experimental cage for 12 min. In the experiment with relieved demonstrators the cage was empty for half of the animals, and it contained empty wire cups for the other half. No effect of cup presence during habituation was observed (Fig. S3c–d, Table S15). In the experiment with hungry demonstrators the cage always contained empty wire cups on the first day of habituation. On habituation days 2 and 3, observers were placed in the experimental cage for 6 min, and the wire cups were introduced for the next 6 min to habituate the observer to their placement during the test.

A glass jar was always placed on the top of the wired cups to prevent the observers from climbing the cups. Demonstrators were placed every day for 10 min in the experimental cage under the wired cups without an observer present. After the last habituation session, animals were placed in separate home cages for 24 h, i.e., until the main test. The relieved/hungry demonstrators were deprived of food immediately after being put in a separate cage, while neutral demonstrators and observers had access to food ad libitum. One hour before the

test, the relieved demonstrators were provided ad libitum access to food. Ten minutes before the test, observers were placed in the testing arena for habituation, and demonstrators were placed under wire cups on the table in the experimental room. Additionally, hungry demonstrators were presented with two chow pellets placed in unreachable distance to the wired cage to induce stress. After habituation, two demonstrators (neutral and relieved or hungry) were placed in the arena for 4 min (under wire cups). Observers who investigated both partners for less than 30 s were excluded from the analysis (for the number of animals excluded based on this criterion, see Table S16). The "relieved" and "hungry" conditions were tested in two consecutive experiments, on separate groups of animals. Number of animals that passed the predefined criteria for "relieved" experiment was n = 12 for females and n = 13 for males, and for "hungry" experiment: n = 8 for females and n = 10 for males.

Data analysis

Distance moved and presence in separate cage compartments in the sCPP test were automatically analyzed using EthoVision XT 15 software (Noldus, The Netherlands). Time spent sniffing relieved/hungry and neutral demonstrators and time spent in the respective zones were scored manually using Boris software[47] by the experimenter, who was blinded to the demonstrators' state. The significance level was set at $p < 0.05$. Comparisons of sample means were performed using analysis of variance (ANOVA) with Geisser-Greenhouse correction followed by Šídák's multiple comparisons test or Student's t-test for cases with only two samples.

Data availability

All data are available at https://zenodo.org/record/6988393. Raw video recordings of the tests will be made available by the corresponding author JRP on request.

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Author contributions

K.M., J.R.P. and Z.H. designed the study. K.M., Z.H., M.K., M.C., Ł.S., A.B. performed experiments. K.M., K.P. and Z.H. analyzed the data. J.R.P. and Z.H. supervised the study. J.R.P., Z.H. and K.M. wrote the paper with contributions from all of the authors.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Information

Prosocial behavior, social reward and affective state discrimination in adult male and female mice

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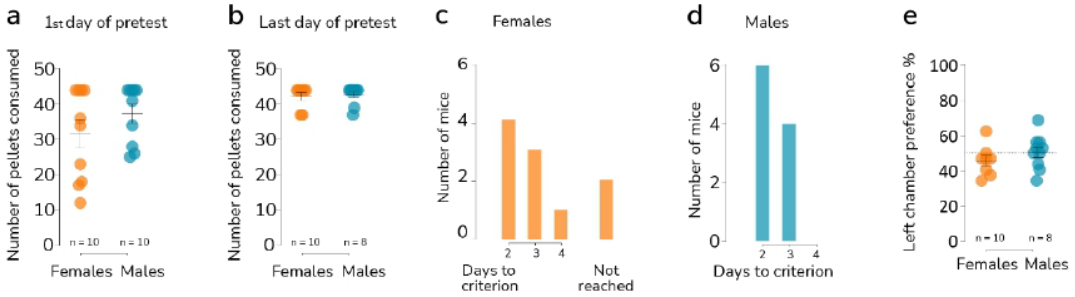
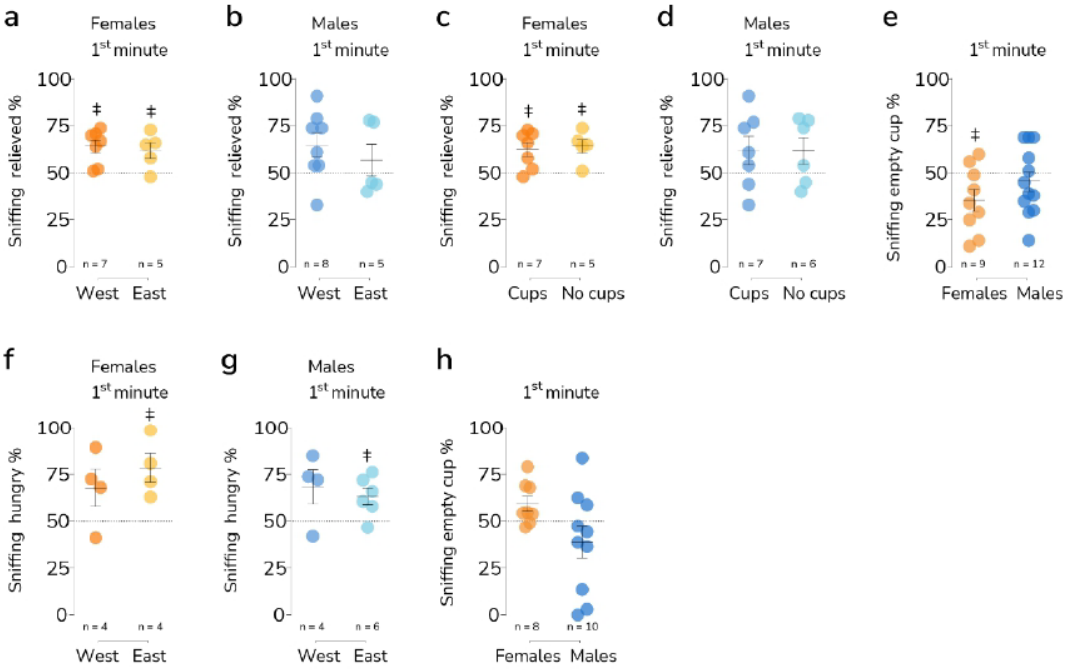
Supplementary Figures

Supplemental Figure S1



Rewards consumed during the pretest phase of the prosocial task. (**a-b**) The number of food pellets consumed by the female and male actors during the first and final days of the pretest, respectively. Each point represents an individual female or male mouse, with the mean and s.e.m. shown in black and the group sizes indicated below. (**c-d**) The number of female and male mice that reached the inclusion criterion of 37 out of 44 food pellets consumed on two consecutive sessions. As shown in (**c**), two female animals did not meet the required criterion. (**e**) No differences form chance level in average preference for the left chamber of the testing apparatus during pretest in female and male mice, respectively.

Supplemental Figure S2



Context effects in the affective state determination task. (**a-b**) No effect of the position of the relieved demonstrator (West or East) on the proportion of the time the observer spent sniffing him in female and male mice, respectively. A significant preference (greater than 0) is indicated by a "ǂ" (one-sample t-test $p<0.05$) (**c-d**) No effect of the presence of the cups during habituation. (**e**) Final day of habituation. No effect of sex on time spent sniffing the empty cup in which the relieved demonstrator was placed during the test. Recordings of final day of habituation of 3 females and 1 male have been lost. (**f-g**) No effect of the position of the hungry demonstrator (West or East) on the proportion of the time the observer spent sniffing him in female and male mice, respectively. (**h**) Final day of habituation. No effect of sex on time spent sniffing the empty cup in which the hungry demonstrator was placed during the test.

Supplementary Tables

Table S1. Prosocial choice test. Number of mice excluded based on predefined criteria.

Sex	Initial n	Number of mice that did not meet criterion		Tested n
		criterion A	criterion B	
females	10	2	0	8
males	10	0	0	10

Criterion A: two days in a row consumed >84% of the reward pellets delivered during the pretest session (37 out of 44)
Criterion B: average pretest side preference below 70%

Table S2. Prosocial choice test. Average pretest and test preference for prosocial compartment.

Fig.	Sex	n	Preference during pretest %		Preference during test %		Mean difference	Paired Student's t-test		
			M	SEM	M	SEM		t	df	p
1e	females	8	44,10	2,70	54,30	3,00	10,16	4,33	7,00	**0,00**
1f	males	10	47,50	2,90	47,80	1,50	0,31	0,10	9,00	0,92

Table S2. Continued. Average preference for the left chamber of the testing apparatus during pretest.

Fig.	Sex	n	M	SEM	One sample t test. Comparison with hypothetical mean (50%)			Mean difference	Unpaired Student's t-test		
					t	df	p		t	df	p
S1e	females	8	45,71	3,07	1,40	7,00	0,20	4,93	1,13	16,00	0,27
	males	10	50,64	3,015	0,21	9,00	0,84				

Table S3. Prosocial choice test. Prosociality score. Females and males comparison.

Fig.	Sex	Prosociality score %[3]									
		n	M	SEM	One sample t test. Comparison with hypothetical mean (0)			Mean difference	Unpaired Student's t-test		
					t	df	p		t	df	p
1g	females	8	10,20	2,30	4,35	7	**0,00**	9,88	2,47	16,00	**0,03**
	males	10	0,30	3,00	0,10	9	0,92				

[3] Mean % of prosocial choices of the last two days of pretest subtracted from mean % of prosocial choices of 4 days of test.

Table S4. Descriptive Statistics and Correlations.

Sex	Behavioral test	Experiment variant	Variable	n	M	SEM	Subject's weight [g][a]			Stimulus' weight [g][b]			Weight difference %[c]		
							r^2	r	p	r^2	r	p	r^2	r	p
females	Prosocial choice test[1]		Prosociality score %	8	10,18	2,34	0,00	0,06	0,88	0,09	0,30	0,46	0,05	-0,22	0,59
	Affective state discrimination test[2]	relieved	Sniffing relieved %	12	63,83	2,72	0,40	-0,62	**0,02**	0,50	-0,70	**0,00**	0,28	0,53	0,07
		hungry	Sniffing hungry %	8	73,10	6,18	0,19	-0,44	0,28	0,00	0,04	0,92	0,48	-0,69	0,06
	Social conditioned place preference test[3]		Preference score [s]	16	285,80	87,07	0,09	0,31	0,24	NA	NA	NA	NA	NA	NA
males	Prosocial choice test[1]		Prosociality score %	10	0,30	3,03	0,13	-0,36	0,38	0,09	0,30	0,30	0,54	-0,73	**0,01**
	Affective state discrimination test[2]	relieved	Sniffing relieved %	13	62,15	4,96	0,07	0,26	0,37	0,05	-0,24	0,42	0,00	0,02	0,93
		hungry	Sniffing hungry %	10	65,22	4,26	0,00	-0,03	0,93	0,04	-0,19	0,59	0,09	0,30	0,39
	Social conditioned place preference test[3]		Preference score [s]	12	221,60	90,59	0,04	-0,12	0,54	NA	NA	NA	NA	NA	NA

1a. Actor's weight on the first day of test; **1b.** Partner's weight on the first day of test; **1c.** Percentage of weight difference between actor and partner on the first day of test. 2a. Observer's weight on the first day of adaptation; 2b. Relieved/hungry demonstrator's weight on the first day of adaptation; 2c. Percentage of weight difference between observer and relieved/hungry demonstrator on the first day of adaptation. 3a. Subject's weight on the day of posttest.

Table S5. Social conditioned place preference test. Average pretest and test preference for social context.

Fig.	Sex	n	Preference during pretest [s]		Preference during test [s]		Mean difference	Paired Student's t-test		
			M	SEM	M	SEM		t	df	p
2a	females	16	893,70	34,85	1040,00	43,56	146,30	2,83	15,00	**0,01**
2b	males	12	855,80	40,59	1010,00	45,28	154,20	4,20	11,00	**0,00**
2d	males	8	918,90	26,18	838,20	58,76	80,69	1,18	7,00	0,28

Table S6. Social conditioned place preference test. Preference score. Females and males comparison.

Fig.	Sex	Social preference score [s][4]								
		n	M	SEM	One sample t test. Comparison with hypothetical mean (0)			Mean difference	Unpaired Student's t-test		
					t	df	p		t	df	p
2c	females	16	285,80	87,07	3,28	15,00	**0,01**	64,20	0,50	26,00	0,62
	males	12	221,60	90,59	2,45	11,00	**0,03**				
2d	males	8	-119,40	117,70	1,02	7,00	0,34				

[4] Time spent in social context pretest [s] subtracted from time spent in social context posttest [s]

Table S7. Affective state discrimination test. Anova with repeated measures table for sniffing demonstrators [s]

Demonstrators state	Fig.	Sex	Source of variation													
			Time					Demonstrator's state					Interaction				
			SS	DF	MS	F	p	SS	DF	MS	F	p	SS	DF	MS	F	p
relieved	3b	females	315,50	3,00	105,20	3,68	**0,02**	106,90	1,00	106,90	2,33	0,14	208,30	3,00	69,44	2,43	0,07
	3c	males	129,50	3,00	43,16	1,25	0,30	584,30	1,00	584,30	6,23	**0,02**	185,90	3,00	61,98	1,80	0,15
hungry	3e	females	49,48	3,00	16,49	0,60	0,57	370,30	1,00	370,30	5,75	**0,03**	425,00	3,00	141,70	5,16	**0,00**
	3f	males	323,10	3,00	107,70	5,53	**0,00**	45,52	1,00	45,52	0,80	0,38	314,80	3,00	104,90	5,39	**0,00**

Table S7. Continued.

Demonstrators state	Fig.	Sex	Comparison of time sniffing relieved vs neutral demonstrators [s]							
			Time [minute]	$M_{relieved}$	$M_{neutral}$	Mean difference	Šídák's multiple comparisons test			
							SE	t	DF	p
relieved	3b	females	1st	17,53	10,46	7,07	2,25	3,14	21,74	**0,02**
			2nd	13,83	12,32	1,52	2,41	0,63	21,19	0,95
			3rd	12,53	12,22	0,31	2,57	0,12	21,96	1,00
			4th	8,95	9,40	-0,45	2,12	0,21	20,55	1,00
	3c	males	1st	20,25	12,17	8,09	2,61	3,09	23,59	**0,02**
			2nd	18,09	12,51	5,59	2,77	2,01	19,25	0,21
			3rd	14,44	13,78	0,65	2,78	0,24	18,91	1,00
			4th	15,61	10,97	4,64	2,84	1,64	19,79	0,39
hungry	3e	females	1st	17,44	5,92	11,53	2,81	4,10	10,62	**0,01**
			2nd	13,10	7,96	5,14	2,97	1,73	11,98	0,37
			3rd	7,72	10,69	-2,97	3,46	0,86	12,37	0,88
			4th	13,09	7,55	5,54	2,84	1,95	13,92	0,26
	3f	males	1st	16,34	8,93	7,41	2,40	3,08	17,21	**0,03**
			2nd	11,88	9,33	2,55	2,17	1,18	17,98	0,69
			3rd	7,79	10,98	-3,19	2,78	1,15	16,55	0,71
			4th	6,72	7,45	-0,73	2,20	0,33	17,99	1,00

Table S8. Affective state discrimination test. Anova with repeated measures table for sniffing demonstrators %

Demonstrators state	Fig.	Source of variation														
		Time					Sex					Interaction				
		SS	DF	MS	F	p	SS	DF	MS	F	p	SS	DF	MS	F	p
relieved	3d	2577,00	3,00	859,00	3,14	**0,04**	138,70	1,00	138,70	0,39	0,54	521,40	3,00	173,80	0,63	0,60
hungry	3g	5341,00	3,00	1780,00	4,01	**0,02**	1134,00	1,00	1134,00	2,05	0,17	784,60	3,00	261,50	0,59	0,63

Table S8. Continued.

Demonstrators state	Fig.	Comparison of time sniffing relieved demonstrators females vs males %							
		Time [minute]	$M_{females}$	M_{males}	Mean difference	Šídák's multiple comparisons test			
						SE	t	DF	p
relieved	3d	1st	63,83	62,15	1,68	5,66	0,30	18,49	1,00
		2nd	52,25	57,77	-5,52	6,28	0,88	22,66	0,86
		3rd	50,00	47,46	2,54	7,37	0,34	22,66	1,00
		4th	50,33	58,46	-8,13	7,82	1,04	20,59	0,77
hungry	3g	1st	73,10	65,22	7,88	7,51	1,05	12,96	0,91
		2nd	65,59	60,97	4,62	7,47	0,62	15,69	0,99
		3rd	46,61	45,80	0,81	13,77	0,06	15,42	1,00
		4th	64,24	45,59	18,65	10,82	1,72	15,97	0,27

Table S9. Affective state discrimination test. One sample t-test for sniffing relieved demonstrators %

Demonstrators state	Fig.	Sex	Sniffing relieved/hungry demonstrators %						
			n	M	SEM	Time [minute]	One sample t test. Comparison with hypothetical mean (50%)		
							t	df	p
relieved	3d	females	12	63,83	2,72	1st	5,08	11,00	**0,00**
				52,25	4,61	2nd	0,49	11,00	0,64
				50,00	4,76	3rd	0,00	11,00	1,00
				50,33	6,30	4th	0,05	11,00	0,96
		males	13	62,15	4,96	1st	2,45	13,00	**0,03**
				57,77	4,265	2nd	1,82	13,00	0,09
				47,46	5,629	3rd	0,45	13,00	0,66
				58,46	4,635	4th	1,83	13,00	0,09
hungry	3g	females	8	73,10	6,18	1st	3,74	7,00	**0,01**
				65,59	5,32	2nd	2,93	7,00	**0,02**
				46,61	10,05	3rd	0,34	7,00	0,75
				64,24	7,33	4th	1,94	7,00	0,09
		males	10	65,22	4,261	1st	3,57	9,00	**0,01**
				60,97	5,238	2nd	2,09	9,00	0,07
				45,80	9,404	3rd	0,45	9,00	0,67
				45,59	7,961	4th	0,55	9,00	0,59

Table S10. Affective state discrimination test. One sample t-test for sniffing relieved demonstrators %.

Demonstrators position (east vs west) comparison.

Demonstrators state	Fig.	Sex	Demonstrator's position	Sniffing relieved/hungry demonstrators %									
				n	M	SEM	One sample t test. Comparison with hypothetical mean (50%)			Mean difference	Unpaired Student's t-test		
							t	df	p		t	df	p
relieved	S2a	females	west	7	64,14	3,47	4,07	6,00	**0,01**	2,14	0,39	10,00	0,70
			east	5	62,00	4,23	2,84	4,00	**0,05**				
	S2b	males	west	8	65,00	6,43	2,33	7,00	0,05	8,20	0,78	11,00	0,45
			east	5	56,80	8,49	0,80	4,00	0,47				
hungry	S2f	females	west	4	67,76	9,97	1,78	3,00	0,17	10,67	0,85	6,00	0,43
			east	4	78,43	7,74	3,67	3,00	**0,03**				
	S2g	males	west	4	68,24	9,20	1,98	3,00	0,14	5,03	0,56	8,00	0,59
			east	6	63,21	4,32	3,06	5,00	**0,03**				

Table S11. Affective state discrimination test. One sample t-test for sniffing relieved/hungry to be demonstrators %.

Demonstrators state	Fig.	Sex	Sniffing relieved/hungry to be demonstrators %									
			n	M	SEM	One sample t test. Comparison with hypothetical mean (50%)			Mean difference	Unpaired Student's t-test		
						t	df	p		t	df	p
relieved	S2e	females	9	35,44	5,83	2,50	8,00	**0,04**	10,06	1,28	19,00	0,22
		males	12	45,50	5,19	0,87	11,00	0,40				
hungry	S2h	females	8	59,32	3,98	2,34	7,00	0,05	20,46	2,01	16,00	0,06
		males	10	38,86	8,50	1,31	9,00	0,22				

Table S12. Groups of animals used in Figures 1-3. Only mice that met predefined criteria.

Sex	Behavioral test	experiment variant	n	Subject's age at the start of the procedure (weeks)[a]			Subject's age at test (weeks)[b]			Subject's weight at the start of the procedure [g][a]		
				M	Range	SEM	M	Range	SEM	M	Range	SEM
females	Prosocial choice test[1]		8	10,30	9,90-10,40	0,06	11,70	11,30-11,90	0,07	19,85	18,60-21,00	0,28
	Affective state discrimination test[2]	relieved	12	12,62	10,90-14,60	0,31	13,09	11,30-15,90	0,36	20,38	18,50-22,20	0,37
		hungry	8	11,49	9,90-13,40	0,52	11,94	10,30-13,90	0,53	21,28	19,40-23,20	0,41
	Social conditioned place preference test[3]		16	10,73	10,60-10,90	0,03	11,56	11,40-11,90	0,05	18,69	17,40-19,60	0,28
males	Prosocial choice test[1]		10	10,30	9,90-11,00	0,14	11,70	11,00-12,40	0,15	26,65	24,10-28,60	0,45
	Affective state discrimination test[2]	relieved	13	11,40	9,90-12,60	0,24	11,95	10,30-14,00	0,30	26,05	23,30-29,10	0,49
		hungry	10	11,52	10,00-14,10	0,43	12,07	10,70-14,60	0,43	26,69	24,80-31,00	0,58
	Social conditioned place preference test[3]		12	10,60	10,00-11,00	0,14	11,60	11,00-12,00	0,14	23,90	21,30-26,00	0,40

Table S12. Continued.

Sex	Behavioral test	Experiment variant	n	Subject's weight at test [g][b]			Stimulus' weight at the start of the procedure [g][c]			Stimulus' weight at test [g][d]		
				M	Range	SEM	M	Range	SEM	M	Range	SEM
females	Prosocial choice test[1]		8	17,01	15,80-18,40	0,34	18,25	16,70-19,90	0,34	15,50	13,8-16,90	0,34
	Affective state discrimination test[2]	relieved	12	19,83	17,20-21,50	0,62	19,68	17,40-21,30	0,42	16,53	15,40-18,70	0,49
		hungry	8	21,70	21,20-22,50	0,22	20,65	19,40-21,60	0,25	17,68	17,20-18,30	0,20
	Social conditioned place preference test[3]		16	19,82	17,40-21,50	0,31	NA	NA	NA	NA	NA	NA
males	Prosocial choice test[1]		10	22,81	20,60-25,40	0,47	25,24	20,60-25,40	0,60	21,40	22,90-27,40	0,45
	Affective state discrimination test[2]	relieved	13	25,42	23,20-28,10	0,51	24,65	21,00-28,20	0,57	20,95	17,30-26,80	0,73
		hungry	10	27,23	24,80-30,20	0,74	25,21	23,40-28-60	0,49	21,56	19,90-23,10	0,65
	Social conditioned place preference test[3]		12	23,74	21,60-25,50	0,34	NA	NA	NA	NA	NA	NA

1a. Actor's age/weight at start of the food deprivation; **1b.** Actor's age/weight on the first day of test; **1c.** Partner's weight on the start of the food deprivation; **1d.** Partner's weight on the first day of test. **2a**. Observer's age/weight on the first day of adaptation; **2b**. Observer's age/weight on test day; **2c**. Relieved/hungry demonstrator's weight on the first day of adaptation. 3a. Subject's age/weight on the day of pretest; **3b**. Subject's age/weight on the day of posttest.

Table S13. Social conditioned place preference test. Conditioning cues.

	Name	Full name	Description/size [mm]	Manufacterer	Website
Bedding types	Aspen 1	ABEDD aspen animal bedding	cubic granulates	Abedd SIA Jelgavas iela 29 Kalnciems, LV-3016, Latvia	https://www.abedd.com/
	Beech 1	Trociny bukowe przesiane gat. 1	shavings	P.P.H. "WO-JAR", Kopernika 3/30, 32-100 Proszowice, Poland	NA
	Beech 2	Trocinka bukowa Facimiech	shavings	PPHU Natur-Drew A. Czaja, os. Kopernika 5/57, 34-100 Wadowice, Poland	NA
	Cellulose	Biofresh Performance Bedding. 1/8' Pelleted Cellulose	pellets	ABSORPTION CORP 6960 Salashan Parkway Ferndale, WA 98248, USA	https://scottpharma.net/product/biofresh-performance-bedding/
Gnawing block types	Block 1	Long thin gnawing block	99 × 19 × 19	Urszula Borgiasz Zoolab, Zielona 14, 28-340 Sedziszow, Poland	http://zoolab.pl/en/enrichment-elements/
	Block 2	Long big gnawing block ("for rats").	99 × 39 × 39		
	Block 3	Cube gnawing block	49 × 39 × 39		

Table S14. Social conditioned place preference test. Number of mice excluded based on predefined criteria.

sCPP protocol	Sex	Initial n	Number of mice that did not meet criterion A	Tested n
6 days	females	17	1	16
6 days	males	12	0	12
2 days	males	8	0	8

Criterion A: Initial preference to any of the context not exceeding 70% in pretest.

Table S15. Affective state discrimination test. One sample t-test for sniffing relieved demonstrators %.

Demonstrators position (cups vs no cups) comparison.

Demonstrators state	Fig.	Sex	Demonstrator's position	Sniffing relieved/hungry demonstrators %									
				n	M	SEM	One sample t test. Comparison with hypothetical mean (50%)			Mean difference	Unpaired Student's t-test		
							t	df	p		t	df	p
relieved	S2c	females	cups	7	62,57	3,75	3,35	6,00	**0,02**	1,63	0,30	10,00	0,77
			no cups	5	64,20	3,73	3,80	4,00	**0,02**				
	S2d	males	cups	7	62,00	7,62	1,57	6,00	0,17	0,33	0,03	11,00	0,98
			no cups	6	61,67	7,13	1,64	5,00	0,16				

In the experiments with hungry demonstrators during the first day of habituation there were always cups in test cage.

Table S16. Affective state discrimination test. Number of mice excluded based on predefined criteria.

Demonstrators state	Sex	Initial n	Number of mice that did not meet criterion A	Tested n
relieved	females	14	2	12
	males	15	2	13
hungry	females	9	1	8
	males	11	1	10

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Adolescent mice exhibit lower reward sensitivity than adults

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Introduction: Adolescence shapes adaptive adult behaviors. It is characterized by increased responsiveness to socially salient stimuli and heightened sensitivity to rewards in peer settings. The particular importance of social context during adolescence indicates that neural circuits responsible for social reward may develop along a different trajectory from those involved in non-social reward processing. However, this remains largely unexplored, as much of the existing research tends to focus on a single reward type, a specific age group of adolescents, or a single sex, thereby limiting a comprehensive understanding of how reward processing evolves across development.

Methods: Here, we investigated how social, cocaine, and palatable food reward sensitivity is expressed in female and male C57BL/6 mice across early- (pubertal onset), mid- (peripubertal phase), and late- (sexual maturity) adolescence, compared to adults. We examined how these different rewards become associated with environmental contexts across developmental stages using the conditioned place preference (CPP) paradigm, a fundamental method for evaluating the motivational properties of stimuli.

Results: We found that adolescent mice exhibited a lower preference for social and palatable food conditioned contexts, while cocaine CPP was not significantly affected by age. Comparisons across CPP tasks confirmed that age, rather than reward type or sex, was the primary factor influencing the magnitude of CPP. Overall, mid- and late-adolescent mice showed reduced mean CPP, with mid-adolescents exhibiting significantly lower odds of expressing a conditioned preference relative to adults.

Discussion: These findings challenge the prevailing assumption that adolescent reward sensitivity universally enhances reward-context learning. Instead, we propose that the attenuated CPP observed in adolescence reflects lower reward sensitivity in emotionally neutral conditions, rather than deficits in associative learning or increased novelty seeking. Our results highlight how developmental stage influences reward-related behaviors and underscore the need for age- and sex-specific analyses in behavioral studies.

KEYWORDS

adolescence, reward sensitivity, conditioned place preference, social reward, cocaine reward, palatable food reward

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Introduction

Adolescence is a crucial transitional phase into adulthood, marked by substantial physical, hormonal, and behavioral changes. It is characterized by heightened sensitivity to environmental influences and increased neural plasticity in brain regions involved in learning, motivation, emotional regulation, and social cognition (Spear, 2000; Knudsen, 2004; Dow-Edwards et al., 2019; Uhlhaas et al., 2023; Baker et al., 2025). While these developmental processes facilitate adaptive growth, they also create vulnerabilities to maladaptive changes, particularly under stress, which may trigger early onset of psychiatric disorders (Spear, 2000; Laviola et al., 2003; Laviola and Marco, 2011; Sheth et al., 2017). Central to these changes is the maturation of the brain's reward system, which underlies increased reward-seeking behavior (Knudsen, 2004; Galván, 2010; Wahlstrom et al., 2010), novelty or sensation seeking (Adriani et al., 1998; Spear, 2000; Laviola et al., 2002; Vetter-O'Hagen and Spear, 2012), risk-taking (Laviola et al., 2003; de Water et al., 2014; Weisfeld and Shattuck, 2017), and susceptibility to drugs of abuse (Schramm-Sapyta et al., 2009; Arguello et al., 2024). It has been consistently reported that gender significantly influences reward-related behaviors (De Cock et al., 2016; Harden et al., 2018; van den Broek et al., 2020; Qutteina et al., 2021) as well as the onset, prevalence, and symptoms of mental disorders (Fairchild, 2011). Teenage girls and boys are more sensitive to social context, process social rewards differently than adults, and generally show heightened sensitivity to all rewards within peer settings (Spear, 2000, 2013; Doremus-Fitzwater et al., 2010). The subjective valuation of rewards undergoes significant changes across development (Csikszentmihalyi et al., 1977; Larson and Richards, 1991; Crone and Dahl, 2012); however, it remains unclear whether sensitivity to various rewarding stimuli (such as social, food, or drug) follows a common developmental pattern. Adolescents exhibit heightened neural responsiveness to socially salient stimuli, which coincides with increased risk-taking behaviors in social settings, including the initiation of substance use, thereby potentially increasing vulnerability to psychiatric disorders (Spear, 2000, 2013; Doremus-Fitzwater et al., 2010). The heightened importance of social cues in adolescence indicates that the development of neural circuits for social rewards may diverge from non-social rewards.

Adolescence and its associated behaviors show notable similarities between humans and laboratory rodents. The ages from 10 to 19 years in humans correspond to postnatal days 28–55 in mice, with both species experiencing similar developmental milestones, including the maturation of the brain's reward system (Piekarski et al., 2017; Weisfeld and Shattuck, 2017). It has been reported that adolescent mice may have increased sensitivity to the rewarding effects of drugs of abuse (Burke and Miczek, 2014), especially under stress conditions (Kreibich et al., 2009; Schindler et al., 2012), and are less sensitive to their aversive effects (Tirelli et al., 2003; Schramm-Sapyta et al., 2009). Adolescent, but not adult mice, were observed to readily develop a preference for a social-conditioned context (Nardou et al., 2019) and demonstrate a stronger motivation to obtain highly palatable food (Friemel et al., 2010; Galván, 2013; Amancio-Belmont et al., 2017; Shteyn and Petrovich, 2025). These findings consistently support the idea that increased reward sensitivity during adolescence may strengthen associative memory, enhance the salience of related cues, and simultaneously facilitate the acquisition of both adaptive and maladaptive behaviors driven by environmental factors such as social experiences, drugs of abuse, or diet. Although this hypothesis is widely accepted, direct empirical evidence remains limited. In both humans and rodents, developmental processes are uneven and dynamic, with females and males maturing at different rates. Consequently, incorporating these factors into experiments is essential to capture and understand behavioral changes accurately. Nardou et al. (2019) demonstrated that conditioned place preference for social reward peaks during late adolescence compared to adulthood in both male and female mice. Additionally, a study on rats reported a sex-independent decline in social reward motivation during mid-adolescence, with both studies emphasizing the importance of multi-timepoint assessments throughout adolescence (Laviola et al., 2003; Tirelli et al., 2003; Murray et al., 2024). Equivalent analyses for other types of rewards are currently lacking. Moreover, despite the common view that adolescence is characterized by heightened reward sensitivity, it was historically debated whether a reduction in reward sensitivity could actually drive increased reward-seeking behaviors, potentially explaining vulnerability to drug abuse in humans (Spear, 2000).

The conditioned place preference (CPP) paradigm is a fundamental method for assessing the rewarding properties of a stimulus. Initially developed to evaluate the reinforcing effects of drugs (Rossi and Reid, 1976; Tzschentke, 1998; Bardo and Bevins, 2000), this approach has been adapted to examine various stimuli, including pain, food, and social cues. In this study, we used modified CPP tasks to measure reward-conditioned preference for social contact, palatable food, or cocaine in male and female C57BL/6 mice at three stages of adolescence: early (~post-natal days 33, indicating puberty onset), mid (~P38, peripubertal period), and late (~P43, reflecting sexual maturity). Contrary to expectations, we observed that reward-conditioned preference was lower in mid-adolescents compared to adults, independent of reward type or sex. This finding challenges the simplistic notion that heightened reward sensitivity during adolescence directly leads to a strengthening of reward-context associations.

Materials and methods

Animals

Experiments were conducted on male and female C57BL/6 mice bred at the animal facility of the Maj Institute of Pharmacology, Polish Academy of Sciences in Krakow. The animals were housed at 22 ± 2 °C, with 40%–60% humidity, and maintained on a 12/12 h light/dark cycle (lights on at 7 AM). Mice had unlimited access to water and food (maintenance chow, 10 mm pellets, Altromin Spezialfutter, cat no. 1324, Germany). After weaning, they were housed in groups of 2–6 littermates per cage in standard Plexiglas cages (length 325 mm × width 170 mm) with wooden blocks for gnawing and nesting material. Female and male mice were kept in separate rooms. All behavioral procedures were performed during the light phase under dim light conditions (5–10 lux) with infrared lighting. Mice were

handled for 3–5 consecutive days before each experiment by placing them in the experimenter's hands for 2–3 min. Every 3 days, animals were marked on their tails for identification. Animals were moved to the experimental room at least 30 min prior to the experiments beginning. The age, sex, and weight of each animal at the start of the experiments are listed in Supplementary Table 1. All procedures were approved by the 2nd Local Bioethics Committee in Krakow (permit numbers 293/2020, 32/2021, 55/2024, 231/2024) and adhered to the guidelines of the European Parliament and the Council of 22 September 2010, on the protection of animals used for scientific purposes (Directive 2010/63/EU and Polish Law Dz.U. 2015 poz. 266). The experiments were planned and reported in accordance with ARRIVE guidelines (Percie du Sert et al., 2020).

Social conditioned place preference

Social CPP was performed as described before (Harda et al., 2022, 2025a,b; Misiołek et al., 2023) and is a revised version of the protocol by Panksepp and Lahvis (2007), with modifications introduced by Dölen et al. (2013), Nardou et al. (2019). Compared to the procedure we described previously, the main difference are the types of beddings. Animals were housed on corn bedding prior to experiments (corn, 2 mm, Rehofix MK2000, Germany). The test was conducted in a custom-made two-chamber apparatus with compartments differing in bedding type (context A: aspen, ABEDD, Latvia or Tapvei GLP, Estonia, context B: 1/8″ Pelleted Cellulose, Scott Pharma Solutions, cat no. L0107, USA) and wooden blocks (context A: cuboid, context B: cube, both from Zoolab, Poland). The social CPP involved three phases: pre-test, conditioning, and post-test (Figure 1A). Behavior during the pre- and post-tests was recorded using a camera (acA1300 – 60 gm, Basler, Germany). During pre-test, mice freely explored the two-chamber cages for 30 min. Mice that spent more than 70% of the time in any context were excluded. To counterbalance contextual bias, half of the animals were assigned to context A as the social-paired environment, while the other half were assigned to context B. The conditioning phase consisted of six consecutive sessions. During the social conditioning session, mice and their littermates were moved to new home cages with one of the conditioning contexts for 24 h. During the isolation session, mice were placed separately in new home cages with the other conditioning context for 24 h. Conditioning started with a social session, and the context alternated daily (social-isolation-social-isolation-social-isolation). The post-test was conducted the day after the last conditioning session. Behavior was automatically analyzed using EthoVision XT 15 software (Noldus, Netherlands). The social place preference index was calculated by subtracting the time spent in the social context during the pre-test from the time spent in the social context during the post-test.

Cocaine conditioned place preference

The cocaine CPP test was conducted using an automatic three-chamber apparatus (Med Associates, St. Albans, VT, USA, MED-CPP-MSAT) as described previously (Sora et al., 1998; Harda et al., 2020, 2025b). The apparatus features two compartments that differ in color and tactile cues, along with a middle compartment that has guillotine doors separating them. Photobeams automatically tracked movement and time spent in each chamber. The CPP paradigm consisted of three phases: pre-test, conditioning, and post-test (Figure 2A). On the first day, the pre-test was conducted to determine the initial preference. Mice were placed in the middle compartment and allowed to explore the entire apparatus for 20 min. Mice that spent more than 70% of their time in one of the conditioning chambers, excluding time in the middle chamber, were excluded. Conditioning sessions took place over the next three days. Each conditioning day included two 40-min sessions: one with saline, and after about 4 h in home cages, a cocaine session (saline-cocaine, saline-cocaine, saline-cocaine). The design was biased; the less preferred chamber was paired with the cocaine injection (cocaine hydrochloride dissolved in saline, i.p., 10 mg/kg, 5 µl/g, cocaine hydrochloride, Toronto Research Chemicals; TRC, Toronto, North York, ON, Canada), while the more preferred chamber was paired with the saline injection (i.p., 5 µl/g, Polpharma, Poland). The post-test was performed in the same way as the pre-test, the day after the last conditioning session. The place preference index was calculated by subtracting the time spent in the cocaine-paired chamber during the pre-test from the time spent in the cocaine-paired chamber during the post-test.

Palatable food conditioned place preference

A palatable food CPP test was conducted in the same experimental setup as the cocaine CPP, using a protocol adapted from Clough et al. (2018). Mice were housed individually and habituated to a palatable food mixture placed in their home cage on a flat glass plate. Each day, they received a fresh portion consisting of 2 pieces of regular chow, 1/3 of an open Oreo cookie (cream side up), 2 Cheetos, and 5 Froot Loops; the nutritional details of these foods are listed in Supplementary Table 2. This habituation process was conducted over two consecutive days prior to the experiment. On the pre-test day, mice were grouped by sex with their littermates in their original home cages (without palatable food, but with chow and water available freely) and allowed to habituate in the experimental room. The pre-test was identical to the cocaine CPP test procedure (Figure 3A). Mice that spent over 70% of their time (excluding time in the middle box) in any conditioning box were excluded from the analysis. An initial bias test was performed, and a biased design was used to assign the less preferred context as the palatable food context (with the food mix) and the more preferred one as the empty cage (without food). Conditioning sessions lasted six consecutive days, with one 60-min session each day. To motivate food-seeking behavior, standard chow was removed from the cages 2 h before and 2 h after each session. Sessions alternated daily, starting with the palatable food session, followed by the empty cage session the next day, and so on (food-empty-food-empty-food-empty). The post-test was then performed, and the place preference index was calculated as in the cocaine CPP test.

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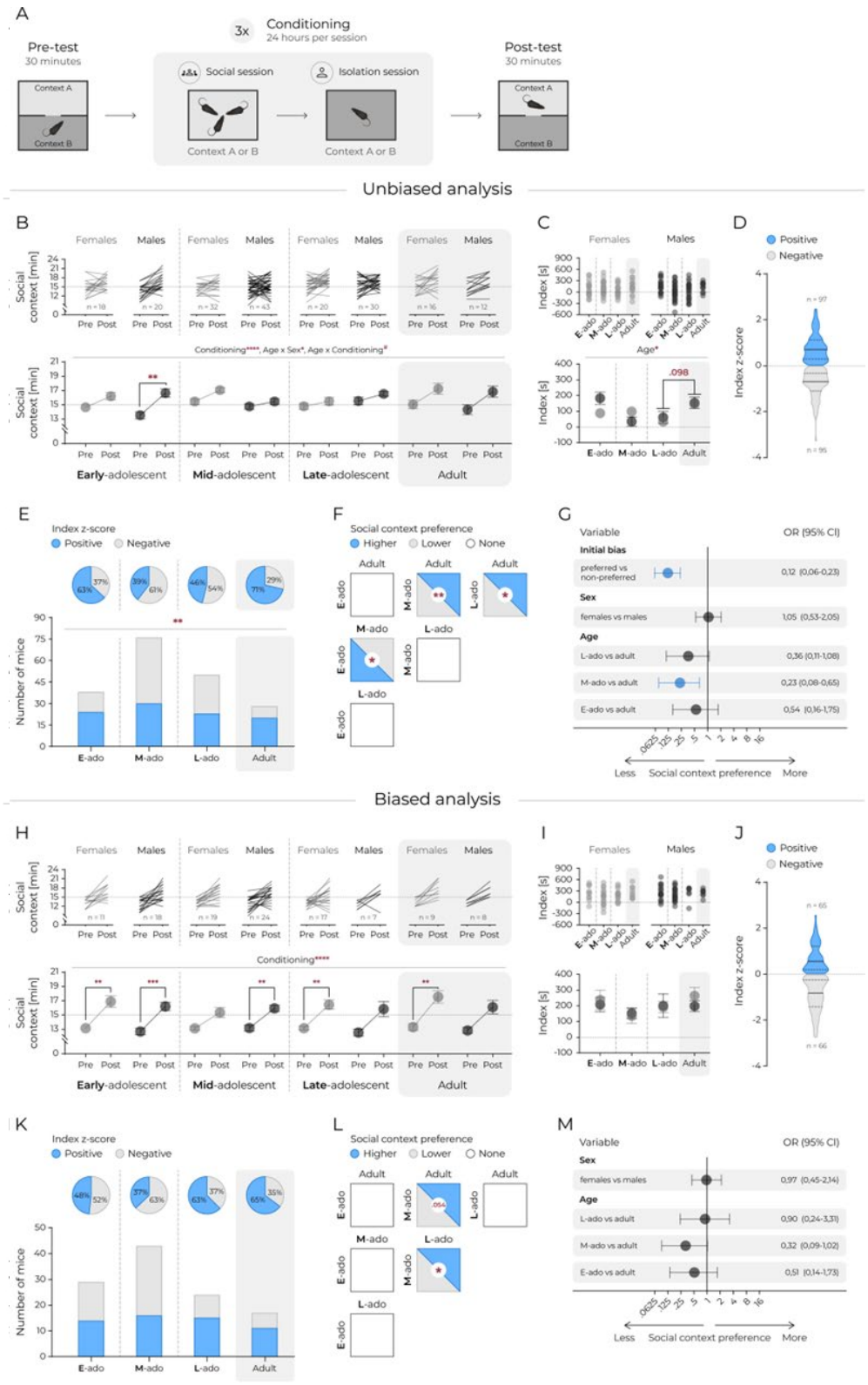

FIGURE 1

Adolescent mice show lower social context preference. **(A)** A schematic representation of the social CPP. **(B)** Time spent in the social context. Top panels: lines represent individual animals. Bottom panel: mean values. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Dotted lines represent random value (i.e., 15 min). Females and males are shown in gray and black respectively. Statistical

Continued

FIGURE 1 (Continued)
analysis was performed using 3-way ANOVA and *post hoc* Tukey's HSD, **corresponds to $p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. **(C)** Difference in seconds between time spent in social context posttest and time spent in social context pretest (index). Top panels: individual animals. Bottom panel: mean values. Whiskers represent s.e.m. values. Dotted lines represent no change. Statistical analysis was performed using 2-way ANOVA and *post hoc* Tukey's HSD, *corresponds to $p \leq 0.05$. **(D)** Distribution of social context preference in the posttest, categorized relative to the group mean (all animals). Sex and age data were combined. Index z-score calculated as: (index of individual animal − mean index of all mice)/standard deviation of index of all mice). Context preference interpreted according to index z-score value: positive, i.e., >0 or negative, i.e., <0. Solid line represents the median, dashed lines mark the quartiles. **(E)** Fractions of animals exhibiting positive (i.e., >0) or negative (i.e., <0) index z-score values are shown. Top panels (pie charts): percent of animals expressing positive (blue) or negative (white) index z-score in each age group. Bottom panels: proportion of animals expressing positive (blue bars) or negative (white bars) index z-score. χ^2 test revealed no significant differences between proportions. **(F)** Pairwise comparisons of social context preference based on the proportion of animals with positive (i.e., >0) and negative (i.e., <0) index z-score values. Each square represents a comparison between each age group. The fill color indicates the predominance of animals expressing higher (blue), lower (gray) social context preference, or no difference (white) between groups. All pairwise χ^2 comparisons were performed, *corresponds to $p \leq 0.05$, **corresponds to $p \leq 0.01$. **(G)** Logistic regression estimates of the effects of initial bias, sex and age on the odds of increased preference for social context based on the index z-score. The circles and horizontal lines indicate the odds ratio and corresponding 95% confidence intervals. Statistically significant effects are marked blue. Panels **(H–M)** correspond to **(B–G)** but with analyses performed only on animals initially preferring the isolation context, i.e., the equivalent of a biased experimental design. **(H)** Time spent in the social context. Top panels: lines represent individual animals. Bottom panel: mean values. Whiskers represent s.e.m. values. Dotted lines represent random value (i.e., 15 min). Females and males are shown in gray and black respectively. Statistical analysis was performed using 3-way ANOVA and *post hoc* Tukey's HSD, **corresponds to $p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. **(I)** Difference in seconds between time spent in social context posttest and time spent in social context pretest (index). Top panels: individual animals. Bottom panel: mean values. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Dotted lines represent no change. Statistical analysis was performed using 2-way ANOVA revealed no significant differences between groups. **(J)** Distribution of social context preference in the posttest, categorized relative to the group mean (all animals). Sex and age data were combined. Index z-score calculated as: (index of individual animal − mean index of all mice)/standard deviation of index of all mice). Context preference interpreted according to index z-score value: positive, i.e., >0 or negative, i.e., <0. Solid line represents the median, dashed lines mark the quartiles. **(K)** Fractions of animals exhibiting positive (i.e., >0) or negative (i.e., <0) index z-score values are shown. Top panels (pie charts): percent of animals expressing positive (blue) or negative (white) index z-score in each age group. Bottom panels: proportion of animals expressing positive (blue bars) or negative (white bars) index z-score. χ^2 test revealed no significant differences between proportions. **(L)** Pairwise comparisons of social context preference based on the proportion of animals with positive (i.e., >0) and negative (i.e., <0) index z-score values. Each square represents a comparison between each age group. The fill color indicates the predominance of animals expressing higher (blue), lower (gray) social context preference, or no difference (white) between groups. All pairwise χ^2 comparisons were performed, *corresponds to $p \leq 0.05$. **(M)** Logistic regression estimates of the effects of sex and age on the odds of increased preference for social context based on index z-score. The circles and horizontal lines indicate the odds ratio and corresponding 95% confidence intervals. There were no statistically significant effects, with one result approaching statistical significance adult vs. M-ado, $p = 0.059$.

Data analysis

All statistical analyses were performed using GraphPad Prism 10.4.2 for Windows (GraphPad Software, n.d.), except for four-way analysis of variance (ANOVA), which was conducted in R (4.5.0) (R Development Core Team, 2025). Animals excluded from experiments based on pre-test criteria (those that spent more than 70% of time in any conditioning context) are listed in Supplementary Table 1. No outliers were detected in the experimental data using the ROUT test. The results were analyzed using ANOVA followed by Tukey's HSD *post hoc* test. Additionally, $\chi 2$ was used to analyze categorical data, and logistic regression was used to assess the effects of initial bias, sex, age, and reward type on the preference to the conditioned context as a binary outcome. Correlations were evaluated using linear regression. Statistical tests and sample sizes are included in the main text or in figure legends.

Results

Adolescent mice show lower social context preference

We first investigated the rewarding effects of social contact using the social CPP test (Figure 1A), in which interaction with a same sex sibling or social isolation was paired with two distinct novel bedding types. An increase in time spent in the social context from pre- to post-test was interpreted as evidence of the rewarding effects of social contact. Across all groups, mice showed a significant increase in time spent in the social context following conditioning (Figure 1B, 3-way ANOVA: $F\text{conditioning}_{(1 \cdot 184)} = 46.53$, $p < 0.0001$). While no main effect of age or sex was detected ($F\text{age}_{(3 \cdot 184)} = 0.67$, $p = 0.555$, $F\text{sex}_{(1 \cdot 184)} = 1.10$, $p = 0.295$) a significant age and sex interaction emerged ($F\text{age}^*\text{sex}_{(3 \cdot 184)} = 3.13$, $p = 0.027$; $F\text{age}^*\text{conditioning}_{(3 \cdot 184)} = 2.54$, $p = 0.058$; $F\text{sex}^*\text{conditioning}_{(1 \cdot 184)} = 0.420$, $p = 0.518$; $F\text{age}^*\text{sex}^*\text{conditioning}_{(3 \cdot 184)} = 1.31$, $p = 0.273$), indicating potential age-dependent differences between males and females. To focus specifically on the effect of the conditioning stimulus and control for initial context preference, we next analyzed the difference in time spent in the social-paired context between post-test and pre-test (i.e., the social preference index; Figure 1C). The advantage of the index over the preference during the post-test (i.e., score) is that it is not confounded by animals that have a consistent preference for the conditioned compartment in both pre- and post-test. We found a significant effect of age (Figure 1C, 2-way ANOVA: $F\text{age}_{(3 \cdot 184)} = 2.97$, $p = 0.033$), but no significant effect of sex or age and sex interaction ($F\text{sex}_{(1 \cdot 184)} = 0.34$, $p = 0.561$; $F\text{age}^*\text{sex}_{(3 \cdot 184)} = 1.66$, $p = 0.179$). While *post hoc* comparisons did not reach statistical significance, a trend toward lower social preference in late-adolescents compared to adults was observed. Given the absence of sex effects, we simplified subsequent analyses by ignoring the sex effect and converting the CPP result to a binary outcome based on the sign of the z-score (i.e., above or below the mean of all cases) (Figure 1D). Cumulative frequency distributions revealed a significant overall difference in social context preference across age groups (Figure 1E, $\chi^2_{(3)} = 11.44$, $p = 0.009$). Specifically,

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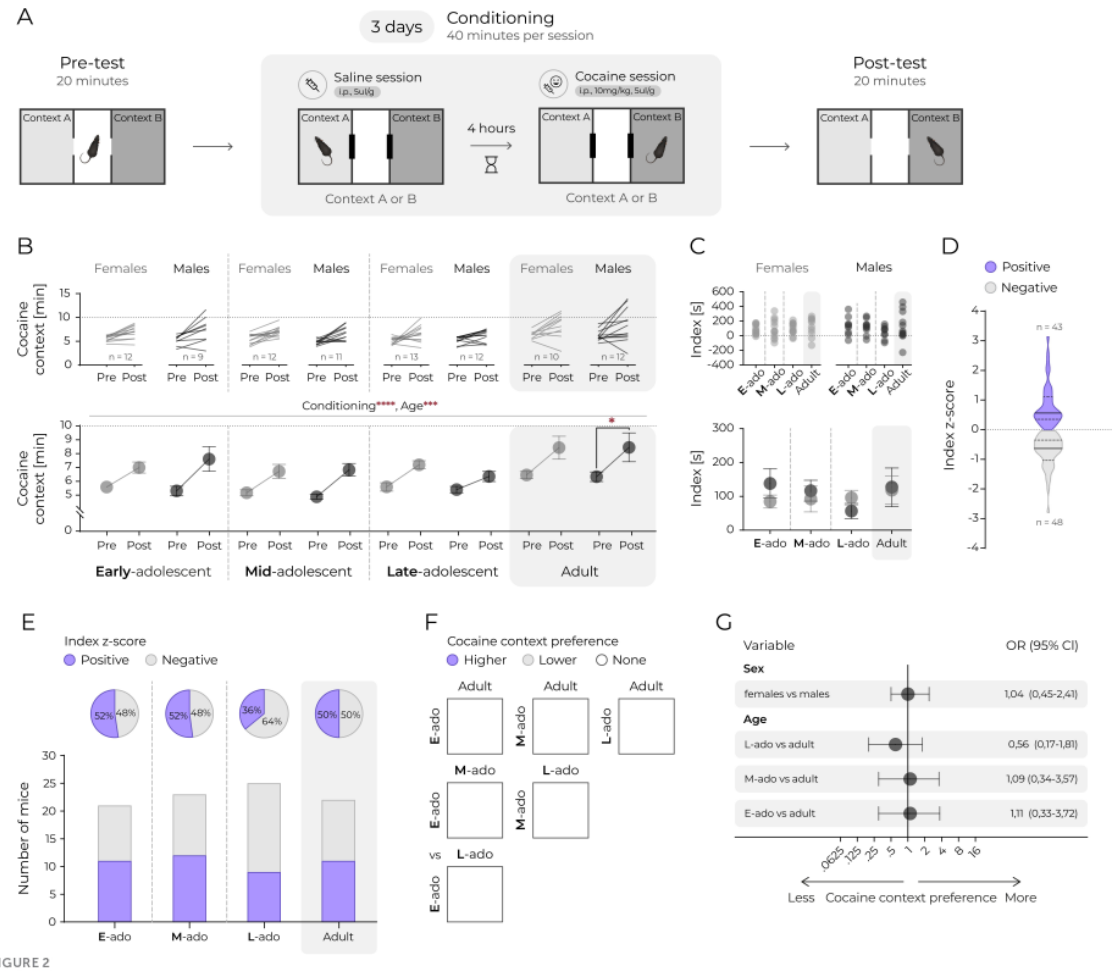

FIGURE 2

Age has no significant effect on cocaine conditioned place preference. **(A)** A schematic representation of the cocaine CPP. **(B)** Time spent in the cocaine context. Top panels: lines represent individual animals. Bottom panel: mean values. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Dotted lines represent random value (i.e., 10 min). Females and males are shown in gray and black, respectively. Statistical analysis was performed using 3-way ANOVA and *post hoc* Tukey's HSD, ***corresponds to $p \leq 0.001$, ****$p \leq 0.0001$. **(C)** Difference in seconds between time spent in cocaine context posttest and time spent in saline context pretest (index). Top panels: individual animals. Bottom panel: mean values. Whiskers represent s.e.m. values. Dotted lines represent no change. Statistical analysis was performed using 2-way ANOVA and revealed no significant differences between groups. **(D)** Distribution of cocaine context preference in the posttest, categorized relative to the group mean (all animals). Sex and age data were combined. Index z-score calculated as: (index of individual animal – mean index of all mice)/standard deviation of index of all mice. Context preference interpreted according to index z-score value: positive, i.e., >0 or negative, i.e., <0. The solid line represents the median, and the dashed lines mark the quartiles. **(E)** Fractions of animals exhibiting positive (i.e., >0) or negative (i.e., <0) index z-score values are shown. Top panels (pie charts): percent of animals expressing positive (purple) or negative (white) index z-score in each age group. Bottom panels: proportion of animals expressing positive (purple bars) or negative (white bars) index z-score. χ^2 test revealed no significant differences between proportions. **(F)** Pairwise comparisons of cocaine context preference based on the proportion of animals with positive (i.e., >0) and negative (i.e., <0) index z-score values. Each square represents a comparison between each age group. The fill color indicates the predominance of animals expressing higher (purple), lower (gray) cocaine context preference, or no difference (white) between groups. All pairwise χ^2 comparisons were not significant. **(G)** Logistic regression estimates of the effects of sex and age on the odds of increased preference for social context based on index z-score. The circles and horizontal lines indicate the odds ratio and corresponding 95% confidence intervals. There were no statistically significant effects.

mid- and late-adolescent mice showed a significantly lower proportion of animals exhibiting social CPP compared to adults (Figures 1E, F, 39% vs. 71%, $\chi^2_{(1)} = 8.37$, $p = 0.004$; 46% vs. 71%, $\chi^2_{(1)} = 4.69$, $p = 0.030$, respectively). Additionally, mid-adolescents showed a significantly lower proportion of social preference compared to early adolescents (Figures 1E, F, 39% vs. 63%, $\chi^2_{(1)} = 5.70$, p = 0.017). To account for potential confounding factors, we conducted a logistic regression analysis that included age, sex, and initial preference bias as predictors of binary CPP expression (Figure 1G). This analysis confirmed that mid-adolescent mice had significantly reduced odds of developing a social context preference relative to adults (95% CI [0.08, 0.65], $p = 0.007$). Importantly, initial preference had a strong influence on CPP expression, with a significant effect observed on the odds of showing a preference post-conditioning (odds ratio 95% CI [0.06, 0.23], $p < 0.0001$).

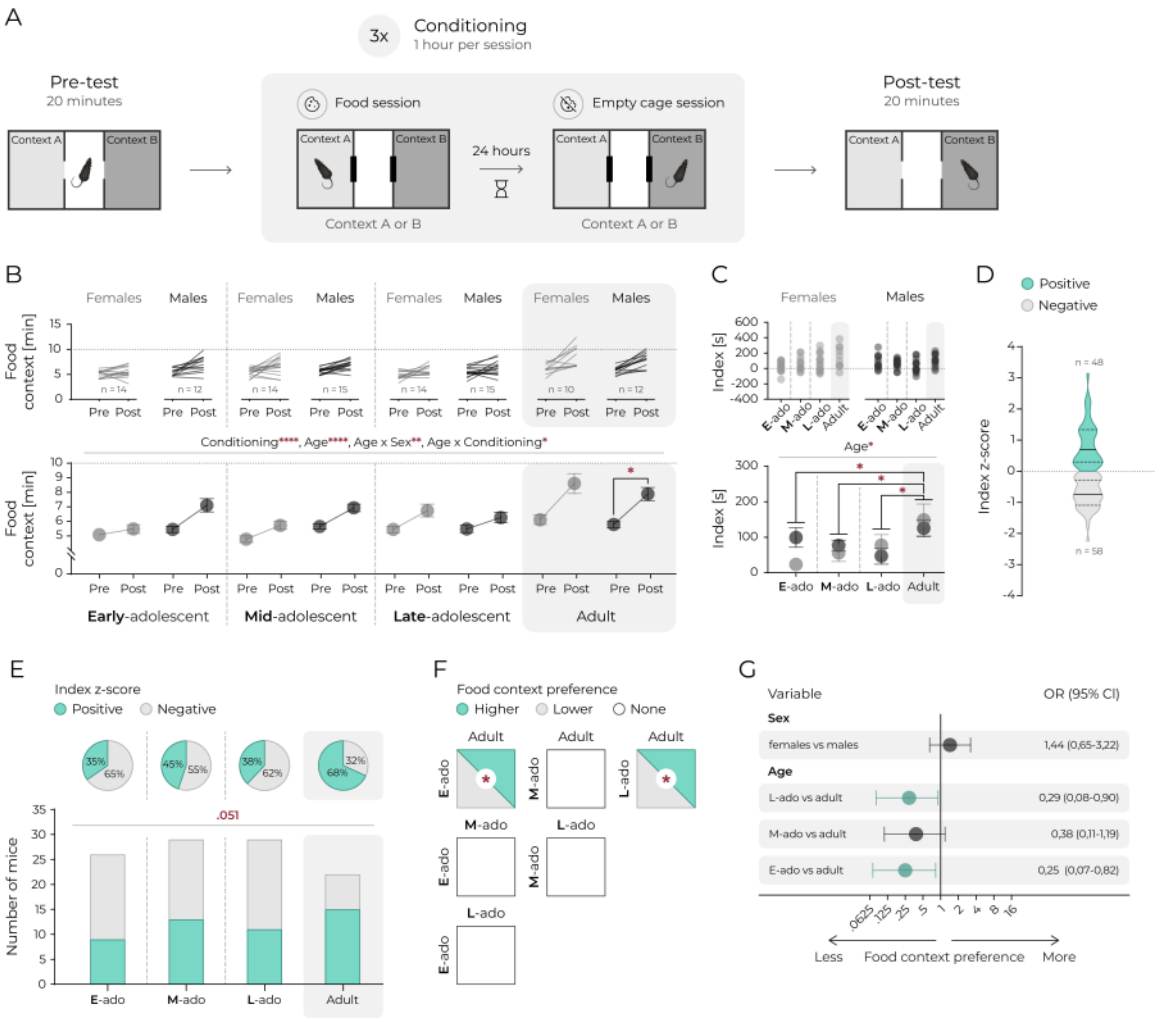

FIGURE 3

Palatable food preference is lower in adolescent mice. **(A)** A schematic representation of the food CPP. **(B)** Time spent in the food context. Top panels: lines represent individual animals. Bottom panel: mean values. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Dotted lines represent random value (i.e., 10 min). Females and males are shown in gray and black respectively. Statistical analysis was performed using 3-way ANOVA and *post hoc* Tukey's HSD, *corresponds to $p \leq 0.05$, **, $p \leq 0.01$, ****$p \leq 0.0001$. **(C)** Difference in seconds between time spent in food context posttest and time spent in empty context pretest (index). Top panels: individual animals. Bottom panel: mean values. Whiskers represent s.e.m. values. Dotted lines represent no change. Statistical analysis was performed using 2-way ANOVA and *post hoc* Tukey's HSD, *corresponds to $p \leq 0.05$. **(D)** Distribution of food context preference in the posttest, categorized relative to the group mean (all animals). Sex and age data were combined. Index z-score calculated as: (index of individual animal − mean index of all mice)/standard deviation of index of all mice). Context preference interpreted according to index z-score value: positive, i.e., >0 or negative, i.e., <0. Solid line represents the median, dashed lines mark the quartiles. **(E)** Fractions of animals exhibiting positive (i.e., >0) or negative (i.e., <0) index z-score values are shown. Top panels (pie charts): percent of animals expressing positive (green) or negative (white) index z-score in each age group. Bottom panels: proportion of animals expressing positive (green bars) or negative (white bars) index z-score. χ^2 test indicated a difference between proportions approaching statistical significance, $p = 0.051$. **(F)** Pairwise comparisons of food context preference based on the proportion of animals with positive (i.e., >0) and negative (i.e., <0) index z-score values. Each square represents a comparison between each age group. The fill color indicates the predominance of animals expressing higher (green), lower (gray) food context preference, or no difference (white) between groups. All pairwise χ^2 comparisons were performed, *corresponds to $p \leq 0.05$. **(G)** Logistic regression estimates of the effects of sex and age on the odds of increased preference for social context based on index z-score. The circles and horizontal lines indicate the odds ratio and corresponding 95% confidence intervals. Statistically significant effects are marked green.

However, analysis of the data revealed that changes in CPP were confounded by the type of bedding used during conditioning. Although the full dataset showed no significant group-level preference for either context before conditioning (cellulose preference 49.95 ± 9%, one-sample *t*-test vs. 50%, $p > 0.05$), age and sex influenced initial context preferences when examined separately. Specifically, a significant effects of sex and an age × sex interaction on baseline preference was observed (Supplementary Figure 1A, 2-way ANOVA $Fage_{(3,21)} = 1.46$, $p = 0.230$, $Fsex_{(1,21)} = 10.82$, $p = 0.001$, $Fage*sex_{(3,21)} = 3.89$, $p = 0.009$). In line with this, the proportion of animals developing a conditioned preference differed across groups: males were more

likely than females to exhibit CPP (Supplementary Figure 1B, 95% CI [1.30, 4.10], $p = 0.005$), and late-adolescents were less likely than adults (95% CI [0.14, 0.97], $p = 0.044$). Logistic regression analysis confirmed that mice that had an initial preference for the social context had significantly higher preference for it in the post-test compared to animals with an initial preference for the isolate context (Figure 1G, 95% CI [0.06, 0.23], $p < 0.0001$). However, conditioning effects (i.e., increase in preference for stimulus-paired context) were only observed in the animals for whom the social interaction was paired with their initially less-preferred context (Supplementary Figure 2A, 4-way ANOVA significant effects: $\text{Finitialbias}_{(4\cdot03)} = 95.0$, $p < 0.001$, $\text{Fconditioning}_{(3\cdot36)} = 52.7$, $p < 0.001$, $\text{Fconditioning*initialbias}_{(3\cdot36)} = 59.4$, $p < 0.001$, $\text{Fsex*age}_{(4\cdot03)} = 3.90$, $p < 0.01$, $\text{Fsex*age*conditioning}_{(3\cdot36)} = 2.66$, $p < 0.01$). Importantly, there was no significant correlation between pre- and post-test time in the social-paired context ($r^2 = 0.02$, $p > 0.05$), suggesting that CPP outcomes were not simply driven by pre-existing preferences. These results reinforce previous findings highlighting the confounding role of reward-independent context biases in CPP paradigms (Tzschentke, 1998; Bardo and Bevins, 2000; Cunningham et al., 2003; Ávila Gámiz et al., 2020; Harda et al., 2022), and contribute to the ongoing debate regarding the use of biased versus unbiased designs (Tzschentke, 1998, 2007; Cunningham et al., 2003; Ávila Gámiz et al., 2020). Therefore, to ensure comparability with the design of cocaine and palatable CPP tests, we restricted subsequent analyses to animals exhibiting no initial preference for the social context.

When the analysis was performed only on animals that had initial preference for the isolate context, we have again found a significant increase in time spent in the social context from pre- to post-test, with no effects of sex, age, or their interactions (Figure 1H, 3-way ANOVA: $\text{Fconditioning}_{(1\cdot105)} = 111.6$, $p < 0.0001$; $\text{Fage}_{(3\cdot105)} = 0.65$, $p = 0.585$; $\text{Fsex}_{(1\cdot105)} = 2.10$, $p = 0.150$; $\text{Fage*sex}_{(3\cdot105)} = 0.991$, $p = 0.400$; $\text{Fage*conditioning}_{(3\cdot105)} = 1.14$, $p = 0.337$; $\text{Fsex*conditioning}_{(1\cdot105)} = 0.072$, $p = 0.788$; $\text{Fage*sex*conditioning}_{(3\cdot105)} = 0.273$, $p = 0.845$). This observation is consistent with the results of the unbiased analysis of the complete set of data. Likewise, there were no significant effects of sex or age on the social preference index, which replicates the observation from the analysis of the complete set (Figure 1I, 2-way ANOVA: $\text{Fage}_{(3\cdot104)} = 1.58$, $p = 0.199$; $\text{Fsex}_{(1\cdot104)} = 0.22$, $p = 0.638$; $\text{Fage*sex}_{(3\cdot104)} = 0.28$, $p = 0.841$). As previously, to simplify further analyses, the effect of sex was excluded, and preference was binarized based on z-score (Figure 1J). Cumulative frequency distributions showed no significant differences in social context preference among age groups (Figure 1K, χ^2 test, $p > 0.05$). Still, a significantly higher proportion of late-adolescent mice preferred the social context compared to mid-adolescents in a pairwise comparison (Figure 1L, 63% vs. 37%, $\chi^2_{(1)} = 3.96$, $p = 0.046$). Logistic regression did not reveal significant effects; the relative odds between adult vs. mid-adolescent mice did not reach significance (Figure 1M, $p = 0.059$). Overall, these findings are consistent with those from the full dataset, although the reduced sample size limited statistical power. Together, both unbiased and biased analyses suggest two separate effects: a lower preference for social-conditioned context in adolescent mice compared to adults, possibly varying across adolescent stages, and a qualitative developmental difference in the proportion of animals developing a preference for the context associated with social contact.

Age has no significant effect on cocaine conditioned place preference

Next, we investigated whether the reduction in social CPP observed during adolescence reflects a social-specific phenomenon or a broader attenuation of reward learning. First, we assessed cocaine-induced CPP, as this paradigm is well-established and cocaine is a drug of abuse known to produce robust CPP with relatively few confounding or aversive effects. Cocaine reward was tested using a three-compartment CPP test with a biased design (Figure 2A). As expected, mice exhibited a significant increase in time spent in the cocaine-paired context (Figure 2B, 3-way ANOVA: $\text{Fconditioning}_{(1\cdot83)} = 67.53$, $p < 0.0001$, $\text{Fage}_{(3\cdot83)} = 6.26$, $p = 0.001$, $\text{Fsex}_{(1\cdot83)} = 0.21$, $p = 0.648$, $\text{Fage*sex}_{(3\cdot83)} = 0.309$, $p = 0.819$, $\text{Fage*conditioning}_{(3\cdot83)} = 0.64$, $p = 0.590$, $\text{Fsex*conditioning}_{(1\cdot83)} = 0.218$, $p = 0.642$, $\text{Fage*sex*conditioning}_{(3\cdot83)} = 0.61$, $p = 0.614$). Although a significant effect of age was detected on the increase in time spent in cocaine-paired context, this effect was not observed when the preference was analyzed using a preference index (Figure 2C, 2-way ANOVA: $\text{Fage}_{(3\cdot83)} = 0.64$, $p = 0.590$, $\text{Fsex}_{(1\cdot83)} = 0.22$, $p = 0.642$, $\text{Fage*sex}_{(3\cdot83)} = 0.61$, $p = 0.614$). Further, when cocaine reward preference was treated as a binary outcome (Figure 2D), comparable proportions of mice across developmental stages exhibited cocaine CPP. Statistical analysis confirmed no significant differences in preference proportions among age groups (Figure 2E, χ^2 test, $p > 0.05$) or in pairwise comparisons (Figure 2F, all χ^2 test, $p > 0.05$). Logistic regression analysis similarly revealed no significant effects of age or sex on cocaine preference (Figure 2G).

Palatable food preference is lower in adolescent mice

To determine whether adolescent reduction in social, but not cocaine CPP, reflects a phenomenon specific to social reward or extends to other natural rewards, we next evaluated CPP using palatable food in a setup similar to cocaine CPP (Figure 3A). This method followed the procedure described by Clough et al. (2018), but without extended chow restriction. Palatable food produced a significant increase in preference for the associated context, with significant interactions between age and sex, and between age and conditioning (Figure 3B, 3-way ANOVA: $\text{Fconditioning}_{(1\cdot98)} = 81.01$, $p < 0.0001$, $\text{Fage}_{(3\cdot98)} = 12.08$, $p < 0.0001$, $\text{Fsex}_{(1\cdot98)} = 3.52$, $p = 0.064$, $\text{Fage*sex}_{(3\cdot98)} = 5.49$, $p = 0.002$, $\text{Fage*conditioning}_{(3\cdot98)} = 3.65$, $p = 0.015$, $\text{Fsex*cond}_{(1\cdot98)} = 0.34$, $p = 0.562$, $\text{Fage*sex*cond}_{(3\cdot98)} = 1.82$, $p = 0.149$). Analysis of preference using index revealed a significant effect of age but no effects of sex or age and sex interaction (Figure 3C, 2-way ANOVA: $\text{Fage}_{(3\cdot98)} = 3.65$, $p = 0.015$, $\text{Fsex}_{(1\cdot98)} = 0.34$, $p = 0.642$, $\text{Fage*sex}_{(3\cdot88)} = 1.82$, $p = 0.149$). Early-, mid-, and late-adolescent mice exhibited significantly lower conditioned preference compared to adults (Figure 3C). When preference, excluding sex as a factor, was categorized as a binary

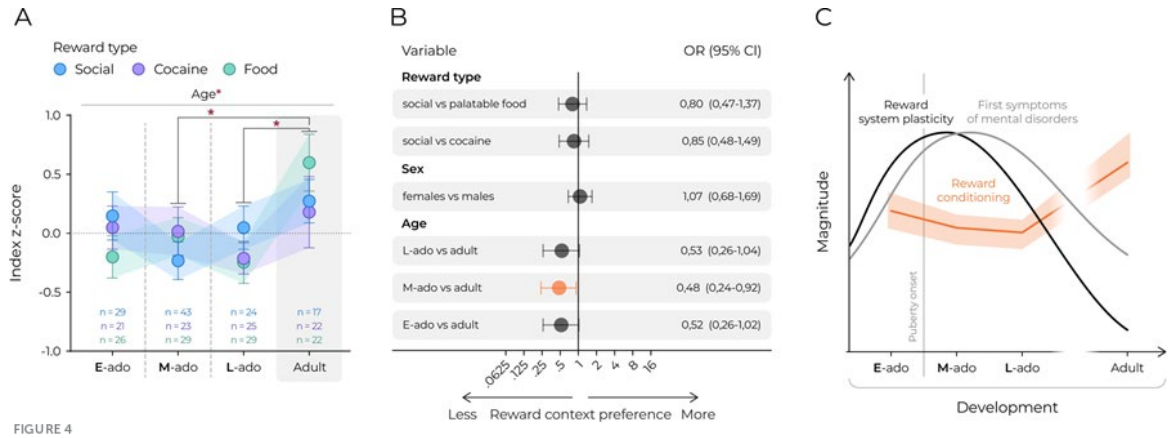

FIGURE 4

Adolescent mice show decreased reward place preference compared to adults. **(A)** Comparison of relative conditioned context preference between social, cocaine and food reward. Sex data were combined. Index z-score calculated for each CPP experiment separately as: (index of individual animal − mean index of all mice)/standard deviation of index of all mice). The colored circles represent mean values, the color-corresponding shadows and whiskers represent s.e.m. values. Statistical analysis was performed using 2-way ANOVA and *post hoc* Tukey's HSD, *corresponds to $p \leq 0.05$. **(B)** Logistic regression estimates of the effects of reward type, sex and age on the odds of increased preference for reward context based on logistic regression. The circles and horizontal lines indicate the odds ratio and corresponding 95% confidence intervals. Statistically significant effects are marked orange. **(C)** A graphical representation of the developmental trajectories of social and non-social conditioned place preference in mice. The orange curve with the color-corresponding shadow represent mean and s.e.m. of reward conditioning (based on Figure 4A). The black and gray lines indicate the sensitive periods for reward system and emergence of first symptoms of mental disorders in humans, respectively [data based on (Uhlhaas et al., 2023)].

outcome (Figure 3D), cumulative frequency analysis showed a trend toward an age effect (Figure 3E, $\chi^2_{(1)} = 3.795$, $p = 0.051$). Pairwise comparisons revealed significantly lower proportions of early- or late-adolescent mice expressing food reward preference relative to adults (Figure 3F, 35% vs. 68%, $\chi^2_{(1)} = 8.37$, $p = 0.004$, 38% vs. 68%, $\chi^2_{(1)} = 4.69$, $p = 0.030$). These differences remained significant in logistic regression analyses (Figure 3G), with odds of preferring the food context were lower by 25% for early-adolescents (95% CI [0.07, 0.82], $p = 0.026$) and by 28% for late-adolescent mice (95% CI [0.08, 0.90], $p = 0.036$) relative to adults. Pre-test time spent in the reward context positively correlated with post-test time for both cocaine ($r^2 = 0.17$, $p < 0.0001$) and palatable food CPP ($r^2 = 0.10$, $p = 0.001$). No evidence suggested that differences in motor activity confounded the results for any reward CPP (Supplementary Figures 3A–D).

Adolescent mice show decreased reward place preference compared to adults

Lastly, we performed a direct comparison of age effects on conditioned preference across all reward types by analyzing normalized z-scores of the preference indices. This approach controlled for variability in the post-test durations and excluded sex as a factor to reduce analytical complexity. There was a significant main effect of age on conditioned preference, but no effects of reward type and no interaction between age and reward type (Figure 4A, 2-way ANOVA: $Freward_{(2,30)} = 0.064$, $p = 0.938$, $Fage_{(3,30)} = 3.21$, $p = 0.023$, $Freward^{*}age_{(6,30)} = 1.07$, $p = 0.381$). Specifically, adult mice showed higher reward CPP compared to mid- and late-adolescents (Figure 4A). In line with these results, logistic regression analysis confirmed a robust age effect as the odds of expressing conditioned preference were decreased by 48% for mid-adolescent (Figure 4B, 95% CI [0.24, 0.92], $p = 0.028$) relative to adult mice, given that the other variables in the model are held constant. No significant main effects of sex or reward type on the conditioned reward preference were identified. To contextualize these developmental changes, Figure 4C presents reward CPP trajectories in relation to puberty onset, the maturation timeline of the reward system, and the period of increased vulnerability to mental disorders, based on Uhlhaas et al. (2023). Although speculative, this comparison suggests that the decrease in CPP observed during mid-adolescence coincides with a period of major neurodevelopmental transition.

Discussion

We found that adolescent mice exhibited weaker reward-conditioned place preference compared to adults, a pattern that was largely consistent across reward types and independent of sex. This result is intuitively at odds with the commonly reported increase in reward sensitivity during adolescence (Spear, 2000, 2013) and in conflict with findings showing robust social CPP in adolescent mice (Nardou et al., 2019).

Conditioned place preference is a well-established method for evaluating the rewarding effects of stimuli; however, outcomes are susceptible to methodological variables, including setup and behavioral parameters, which can significantly influence results (Bardo and Bevins, 2000; Cunningham et al., 2003; Ávila Gámiz et al., 2020; Yates, 2023). One critical factor is initial context bias, which can substantially shape CPP expression (Tzschentke, 1998; Cunningham et al., 2003). Although an unbiased design allows for the detection of both appetitive and aversive responses, the strength of CPP can vary substantially across stimuli (Kummer et al., 2014),

and even subtle contextual preferences may obscure reward-driven learning. To address this, we followed task-specific designs, an unbiased design for social CPP (Harda et al., 2022, 2025a; Misiołek et al., 2023), a biased design for cocaine CPP (Harda et al., 2020), and adhered to the original, biased protocol for palatable food CPP (Clough et al., 2018). To account for potential bias-related effects, we analyzed both biased and unbiased versions of social CPP and found consistent results. As emphasized by Yates (2023), the interpretation of CPP depends critically on both experimental design and the choice of behavioral metrics, typically the index (change from pre- to post-test) or the score (post-test difference in preference between compartments). In our study, animals that already exhibited a strong initial preference for the social context showed no further increase following conditioning, and all animals preferred the social context at post-test, irrespective of initial bias, suggesting potential ceiling effects. Analyses conducted on both the full dataset and the biased subset of social CPP data yield consistent results: the effects of age and no effect of sex, although the specific behavioral metrics reaching statistical significance varied between analyses. Finally, we have to emphasize that our study was not designed to directly compare the sensitivity to social versus non-social rewards. Evaluation of differences in motivational value between reward types requires concurrent conditioning paradigms and assessment of how preferences shift as a function of parameters such as dose [e.g., (Kummer et al., 2014)]. We cannot exclude the possibility that different doses of cocaine might have produced different magnitudes of CPP, or that longer periods of restricted food access could have enhanced the motivational salience of palatable food reward. In our protocol, food deprivation was deliberately minimized to reduce such confounds. Moreover, while social CPP was based on a two-chamber design, palatable food and cocaine CPP used a three-chamber apparatus, which precludes direct comparison of times spent in the stimulus-paired context. For these reasons, we focused not on the absolute magnitude of CPP between reward types, but rather on the developmental trajectories and the influence of sex and age, the factors that are not inherently test-type dependent.

Our social CPP findings align with recent work by Murray et al. (2024), who reported a decrease in social reward motivation in mid-adolescent rats using operant conditioning, although with the highest motivation observed in early adolescents. In contrast, Nardou et al. (2019) have elegantly demonstrated in the social CPP task that social contact is rewarding in male and female adolescent but not adult mice, a result we have previously replicated (Harda et al., 2022). However, when we extended conditioning and used siblings as social partners, we observed robust social CPP in 14-week-old adult mice. This aligns with the role of social context, prior social experiences, internal state, and kinship in social CPP (Panksepp and Lahvis, 2007; Cann et al., 2020; Harda et al., 2022; Misiołek et al., 2023). In humans, adolescents show transient increases in social motivation toward non-kin peers rather than family members (Spear, 2000, 2013), which may explain why social CPP could be enhanced during adolescence when conditioned with unfamiliar conspecifics. In the present study, we found no consistent sex differences in social CPP, in line with several studies (Panksepp and Lahvis, 2007; Nardou et al., 2019; Murray et al., 2024), though others reported higher social CPP in male rats compared to females (Douglas et al., 2004; Grotewold et al., 2014) or higher social CPP in female mice compared to males (Cann et al., 2020), depending on housing and prior social isolation.

For palatable food CPP, our findings contrast with multiple reports showing enhanced food intake and motivation during adolescence (Friemel et al., 2010; Galván, 2013; Amancio-Belmont et al., 2017; Shteyn and Petrovich, 2025), especially during mid-puberty (Friemel et al., 2010). Shteyn and Petrovich (2025), for example, found that adult rats prefer palatable food regardless of satiety, whereas adolescents do so only when satiated. Moreover, prior studies showed that adult mice form CPP for high-fat/sugar food mixes but not for chow alone (Clough et al., 2018), suggesting that the nutritional composition, rather than caloric content *per se*, drives food reward. In our study, we imposed a mild (2-h) food deprivation prior to conditioning, aiming to enhance motivation while minimizing stress. However, this protocol may have differentially affected motivation: adults may have selectively consumed more rewarding components, strengthening context-reward associations, whereas adolescents may have consumed food more evenly, leading to weaker CPP. Food consumption was not directly monitored, which limits this interpretation. As with social CPP, we found no consistent sex effects. Apparent differences between early-adolescent males and females were attributable to increased time spent in the neutral compartment by females, likely reflecting anxiety-like behavior. This lack of robust sex effects aligns with previous findings (Anderson and Petrovich, 2015, 2018), though others have reported stronger food CPP in females (Sinclair et al., 2017; Shteyn and Petrovich, 2025; Shteyn et al., 2025).

Adolescent drug-related reward sensitivity is often linked to heightened stress responsivity (Burke and Miczek, 2014) or reduced sensitivity to drug aversiveness (Tirelli et al., 2003; Schramm-Sapyta et al., 2009; Doremus-Fitzwater and Spear, 2016). Our experiments were conducted under low-stress conditions using mice bred on-site, and cocaine was selected due to its reliable CPP-inducing effects and relatively low aversive profile. Estrous cycles were not monitored, limiting interpretation of sex effects, especially given the known influence of estrogen and progesterone on cocaine reward (Evans and Foltin, 2010; Peart et al., 2022). Furthermore, we did not assess dose-response effects, leaving open the possibility that age-related differences in CPP may emerge at other doses. Despite these limitations, we observed significantly lower CPP in adolescent mice compared to adults, regardless of sex or the type of reward. This indicates that developmental changes in reward sensitivity may be more complex than previously assumed. A similar finding was reported by Laviola's group, where adolescent mice, within an age range comparable to the early-to-late adolescent period examined in our study, exhibited increased impulsivity and restlessness, along with reduced or absent rewarding effects of amphetamine compared to young adult mice (> postnatal day 60) (Adriani and Laviola, 2003; Tirelli et al., 2003). While some earlier studies have reported heightened reward motivation during adolescence, many were not explicitly designed to assess reward sensitivity. In contrast, our results provide strong evidence that reward-context associations, as measured by CPP, are diminished during adolescence in specific experimental contexts, highlighting a more nuanced developmental trajectory of reward processing

Conditioned place preference is influenced by four main factors: reinforcement, which reflects the intrinsic rewarding properties of the stimulus; motivational state, encompassing

the animal's drive for reward-seeking; memory, involving the acquisition and recall of the context-reward association; and the conditioned response, representing behavioral changes following conditioning (McKendrick and Graziane, 2020). These individual contributions are difficult to disentangle within the CPP outcome, meaning that lower CPP in adolescence arises from changes in one or more of these domains. Learning impairments, however, appear unlikely. We previously demonstrated that adolescent mice develop social CPP following shorter conditioning than adults (Harda et al., 2022; Misiołek et al., 2023), and our current and past findings demonstrate robust cocaine CPP across age groups (Harda et al., 2025b), suggesting intact or even enhanced learning capacity during adolescence. Moreover, we found no evidence of increased novelty-seeking overriding conditioned responses. Although adolescents spent more time in the neutral compartment in both food and cocaine tasks, this did not co-occur with increased exploratory behavior and likely reflects baseline behavioral differences (see Supplementary Figure 4). Taken together, these findings support the interpretation that the attenuated CPP observed during adolescence is more likely due to decreased reward sensitivity or motivation rather than deficits in learning or an increase in novelty-seeking behavior.

From the perspective of behavioral learning theory, behavior is shaped through the reinforcement and punishment of actions (Vargas, 2017; Schlinger, 2021). Therefore, lower CPP in adolescents may reflect either diminished reinforcement or a reduced aversiveness of stimulus absence, or both, which together weaken associative learning. This idea aligns with neurodevelopmental research, which shows that mid-adolescence is a period of increased brain plasticity and heightened emotional reactivity (Spear, 2000; Uhlhaas et al., 2023). However, these factors alone do not fully explain the lowered reward preference we found. In humans, adolescence is marked by a stronger influence of emotional biases on decision-making and executive functions (Spear, 2013; Poon, 2017). Executive functions encompass both "cold" processes, such as logical thinking and control, and "hot" processes that involve emotions, impulsivity, and reward sensitivity, which peak during mid-adolescence (Poon, 2017). Thus, we propose that it is a developmental stage characterized by generally lower baseline reward sensitivity, unless the stimuli are highly emotional or socially salient. A similar interpretation was previously proposed by Laviola et al.'s (2003) group, who reported that during adolescence, dopamine release is lower at baseline but higher after stimulation due to a larger dopamine storage pool compared to adults (Tirelli et al., 2003). This may serve as a gating mechanism to prevent unnecessary experience-dependent plasticity, which could otherwise contribute to the emergence of psychiatric disorders (Fairchild, 2011; Galván, 2013). Understanding these developmental changes is crucial for developing strategies to prevent mental health disorders that begin in adolescence and persist into adulthood. Future research should examine how the social environment, stress, hormones, and individual behavior affect reward sensitivity and vulnerability to psychiatric disorders during this critical period. Insights from such studies could inform more effective, targeted pharmacological and psychological treatments for adolescent mental illnesses.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was approved by the 2nd Local Bioethics Committee in Krakow. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. MC: Investigation, Writing – review & editing. MK: Investigation, Writing – review & editing. AR: Investigation, Writing – review & editing. JN: Investigation, Writing – review & editing. BZ: Conceptualization, Writing – review & editing. ŁS: Investigation, Writing – review & editing. MK-J: Investigation, Writing – review & editing. ZH: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. JRP: Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2025.1695375/full#supplementary-material

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Supplementary Material

Adolescent mice exhibit lower reward sensitivity than adults

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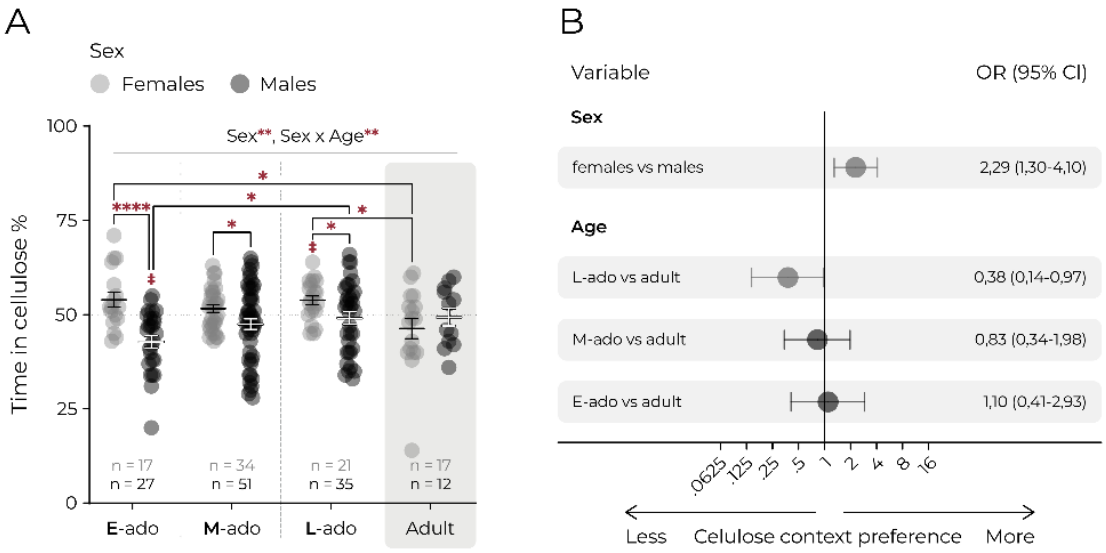
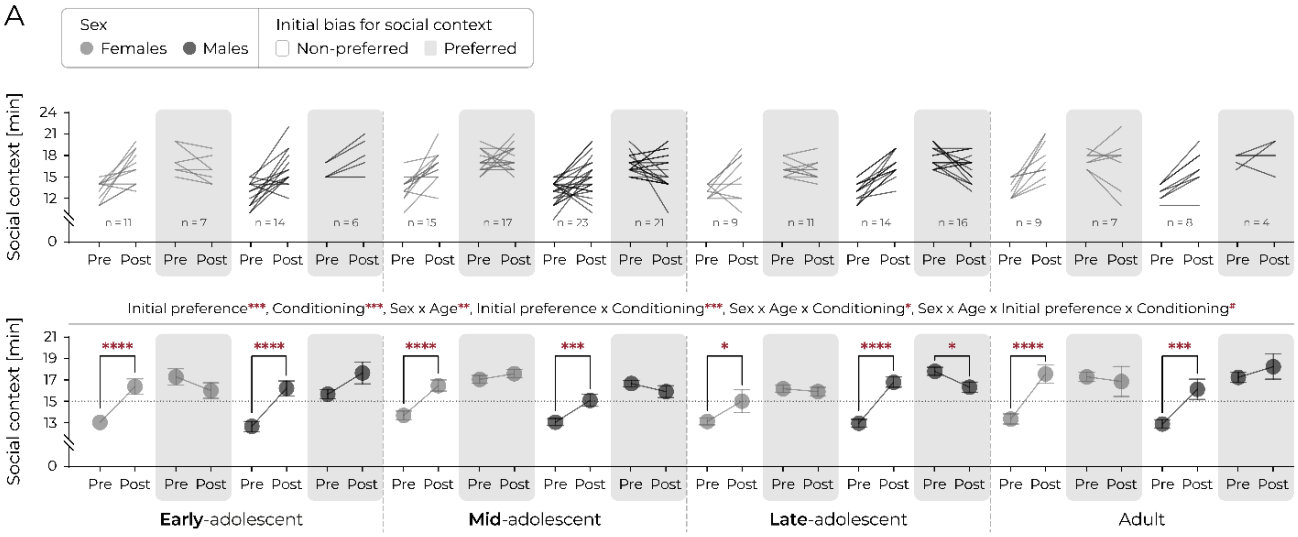


Supplementary Figure 1. Mice express significant bias in initial preference in pretest before place conditioning.

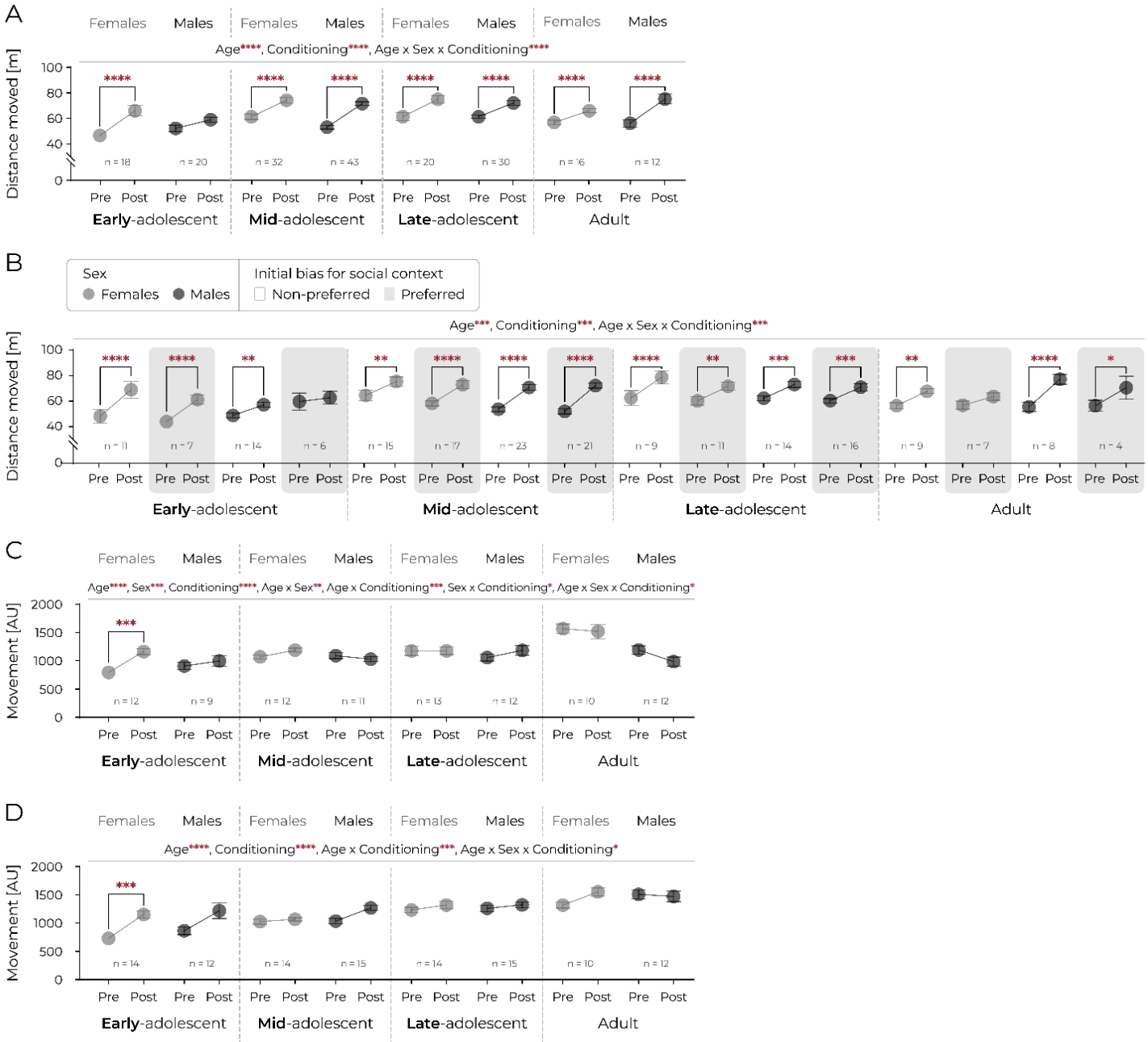
(A) Time spent in the cellulose context in pretest. Graph includes data from all animals, including animals later excluded based on criterion of initial context bias (>70% of time in any of the contexts in pretest). Each circle represents an individual animal. Whiskers represent mean and s.e.m. values. Dotted line represents random value (i.e. 50%). Females and males are shown in gray and black respectively. Statistical analysis was performed with 2-way ANOVA: $Fage_{(3,21)} = 1.46$, $p = .230$, $Fsex_{(1,21)} = 10.82$, $p = .001$, $Fage*sex_{(3,21)} = 3.89$, $p = .009$ and Tukey's HSD, "*" corresponds to $*p \leq .05$, $**p \leq .01$, and $****p \leq .0001$ and "#", $p \leq .08$.

(B) Logistic regression estimates of the effects of sex and age on the odds of increased preference for cellulose context based on the index z-score. The circles and horizontal lines indicate the odds ratio and corresponding 95% confidence intervals. Statistically significant effects are marked gray. The odds of cellulose bedding preference in the pretest was 29% higher for females compared to males (95% CI [1.23, 4.09], $p = .0046$) and 37% lower for late-adolescent mice compared to adults (odds ratio 95% CI [.14, .97], $p = .045$).



Supplementary Figure 2. Increase in place preference after conditioning differs as a function of initial bias for social context.

(A) Time spent in the social context, according to initial bias. Top panels: lines represent individual animals. Bottom panel: mean values. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Dotted lines represent random value (i.e. 15 min). Females and males are shown in gray and black respectively. Females and males are shown in dark gray and black respectively. Bias for social context for initially non-preferred and preferred context are marked white and light gray background, respectively. Statistical analysis was performed using 4-way ANOVA with matched values: : $F_{initialbias(4.03)} = 95.0$, $p < .001$, $F_{conditioning(3.36)} = 52.7$, $p < .001$, $F_{sex(4.03)} = .49$, $p = .486$, $F_{age(4.03)} = 1.02$, $p = .386$, $F_{sex*age(4.03)} = 3.90$, $p = .010$, $F_{sex*initialbias(4.03)} = 1.47$, $p = .227$, $F_{age*initialbias(4.03)} = .04$, $p = .990$, $F_{sex*conditioning(3.36)} = .41$, $p = .523$, $F_{age*conditioning(3.36)} = 1.85$, $p = .140$, $F_{conditioning*initialbias(3.36)} = 59.44$, $p < .001$, $F_{sex*age*initialbias(4.03)} = .58$, $p = .630$, $F_{sex*age*conditioning(3.36)} = 2.66$, $p = .050$, $F_{sex*initialbias*conditioning(3.36)} = .06$, $p = .806$, $F_{age*initialbias*conditioning(3.36)} = .63$, $p = .599$, $F_{sex*age*initialbias*conditioning(3.36)} = 2.61$, $p = .053$, and post hoc Tukey's HSD, '*' corresponds to $p \leq .05$, '**', $p \leq .01$, '***', $p \leq .001$, '****' $p \leq .0001$.


A
Females
Males
Age****, Conditioning****, Age x Sex x Conditioning****
Distance moved [m]
Early-adolescent
Mid-adolescent
Late-adolescent
Adult
B
Sex
Females
Males
Initial bias for social context
Non-preferred
Preferred
Age***, Conditioning***, Age x Sex x Conditioning***
Distance moved [m]
Early-adolescent
Mid-adolescent
Late-adolescent
Adult
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Age****, Sex***, Conditioning****, Age x Sex**, Age x Conditioning***, Sex x Conditioning*, Age x Sex x Conditioning*
Movement [AU]
Early-adolescent
Mid-adolescent
Late-adolescent
Adult
D
Age****, Conditioning****, Age x Conditioning***, Age x Sex x Conditioning*
Movement [AU]
Early-adolescent
Mid-adolescent
Late-adolescent
Adult

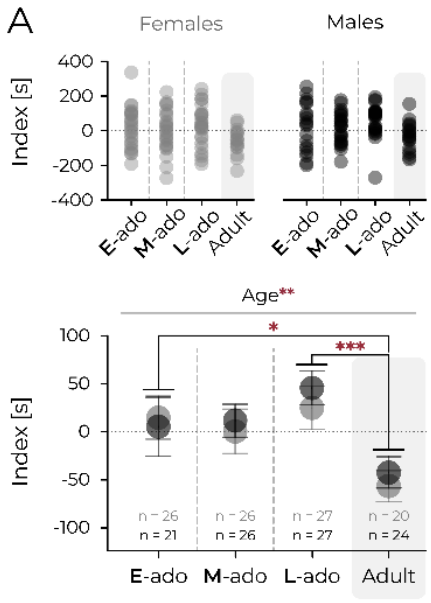
Supplementary Figure 3. Analysis of motor activity during the pre-test and posttest phases in social, cocaine, and food CPP paradigms revealed no evidence of a confounding effect on context preference.

(A) Distance moved during the pre- and post-test session in unbiased social CPP. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Females and males are shown in gray and black respectively. Statistical analysis was performed using 3-way ANOVA with matched values was performed: $F_{conditioning(1.18)} = 242.4$, $p < .0001$, $F_{age(3.18)} = 11.16$, $p < .0001$, $F_{sex(1.18)} = .35$, $p = .554$, $F_{age*sex(3.18)} = 1.80$, $p = .147$, $F_{age*conditioning(3.18)} = 1.11$, $p = .346$, $F_{sex*conditioning(1.18)} = .420$, $p = .994$, $F_{age*sex*conditioning(3.18)} = 7.45$, $p < .0001$ and post hoc Tukey's HSD, '****' corresponds to $p \leq .0001$.

(B) Distance moved during the pre- and post-test session, according to initial bias in social CPP. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Females and males are shown in dark gray and black respectively. Bias for social context for initially non-preferred and preferred context are marked white and light gray background, respectively. Statistical analysis was performed using 4-way ANOVA with matched values: $F_{conditioning65.64)} = 214.03$, $p < .0001$, $F_{age(184.40)} = 9.62$, $p < .001$, $F_{sex(184.40)} = .09$, $p = .759$, $F_{initialbias(184.40)} = 1.26$, $p = .263$, $F_{sex*age(184.40)} = 2.06$, $p = .111$, $F_{sex*initialbias(184.40)} = 2.63$, $p = .107$, $F_{sex*conditioning(65.64)} = .06$, $p = .812$, $F_{age*conditioning(65.64)} = 1.17$, $p = .321$, $F_{conditioning*initialbias(65.64)} = 1.58$, $p = .210$, $F_{age*sex*conditioning(65.64)} = 7.06$, $p < .001$, $F_{sex*initialbias*conditioning(65.64)} = .00$, $p < .953$, $F_{age*initialbias*conditioning(65.64)} = 1.63$, $p < .183$, $F_{age*sex*initialbias(184.40)} = .94$, $p = .420$, $F_{age*sex*initialbias*conditioning(65.64)} = .22$, $p = .885$ and post hoc Tukey's HSD, '*' corresponds to $p \leq .05$, '**', $p \leq .01$, '***', $p \leq .001$, '****' $p \leq .0001$.

(C) Distance moved during the pre- and post-test session in cocaine CPP. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Females and males are shown in gray and black respectively. Statistical analysis was performed using 3-way ANOVA with matched values was performed: $F_{conditioning(1.18)} = 3.62$, $p = .060$, $F_{age(3.83)} = 10.78$, $p < .0001$, $F_{sex(1.18)} = 12.33$, $p = .001$, $F_{age*sex(3.83)} = 5.43$, $p = .002$, $F_{age*conditioning(3.82)} = 7.69$, $p = .0001$, $F_{sex*conditioning(1.18)} = 5.82$, $p = .018$, $F_{age*sex*conditioning(3.83)} = 3.03$, $p = .034$ and post hoc Tukey's HSD, '*' corresponds to $p \leq .05$, '**', $p \leq .01$, '***', $p \leq .001$, '****' $p \leq .0001$.

(D) Distance moved during the pre- and post-test session in food CPP. Circles and connecting lines represent means and matched values. Whiskers represent s.e.m. values. Females and males are shown in gray and black respectively. Statistical analysis was performed using 3-way ANOVA with matched values was performed: $F_{conditioning(1.98)} = 39.48$, $p < .0001$, $F_{age(3.98)} = 27.29$, $p < .0001$, $F_{sex(1.98)} = 3.20$, $p = .076$, $F_{age*sex(3.98)} = .33$, $p = .801$, $F_{age*conditioning(3.98)} = 6.77$, $p = .0003$, $F_{sex*conditioning(1.98)} = .59$, $p = .445$, $F_{age*sex*conditioning(3.98)} = 2.83$, $p = .042$ and post hoc Tukey's HSD, '*' corresponds to $p \leq .05$, '***', $p \leq .001$, '****' $p \leq .0001$.



Supplementary Figure 4. Adult mice, regardless of sex, spent less time in the neutral context than juvenile mice after conditioning. Related to Figures 2C and 3C.

(A) Difference in seconds between time spent in neutral context posttest and time spent in neutral context pre-test (index). Cocaine and food CPP data were combined. Top panels: individual animals. Bottom panel: mean values. Whiskers represent s.e.m. values. Dotted lines represent no change. Statistical analysis was performed using 2-way ANOVA: $Fage_{(3.19)} = 5.43$, $p = .0001$, $Fsex_{(1.19)} = .37$, $p = .542$, $Fage*sex_{(3.19)} = .18$, $p = .907$ and post hoc Tukey's HSD, '*' corresponds to $p \leq .05$, '**', $p \leq .01$.

Supplementary Table 1 Age, weight and number of excluded animals in all CPP tests.

CPP test	Sex	Age bin	Excluded n[a]	Tested n	Subject's weight at post-test [g]			Subject's age at post-test (days)		
					Range	Mean	SEM	Range	Mean	SEM
Social (unbiased)	Females	Early-adolescent	1	18	4,5	13,9	0,3	2	31	0,1
		Mid-adolescent	0	32	4,5	15,2	0,2	5	37	0,4
		Late-adolescent	0	20	2,7	15,5	0,2	4	44	0,4
		Adult	1	16	4,1	19,8	0,3	2	82	0,2
	Males	Early-adolescent	1	20	4,2	17,6	0,3	2	32	0,1
		Mid-adolescent	3	44	8,8	17,4	0,4	4	37	0,3
		Late-adolescent	0	30	6,7	19,1	0,3	6	42	0,3
		Adult	0	12	3,9	23,7	0,3	8	83	0,9
Social (biased)	Females	Early-adolescent	-	11	5,2	16,3	0,7	3	32	0,3
		Mid-adolescent	-	19	7,4	17,1	0,6	5	37	0,5
		Late-adolescent	-	17	7,2	18,3	0,6	6	42	0,4
		Adult	-	9	2,9	20,1	0,3	2	82	0,2
	Males	Early-adolescent	-	18	7,4	16,1	0,5	2	32	0,1
		Mid-adolescent	-	24	11,7	15,6	0,5	4	36	0,3
		Late-adolescent	-	7	6,3	18,0	1,0	5	43	0,6
		Adult	-	8	3,9	23,6	0,5	8	83	1,2
Cocaine	Females	Early-adolescent	1	12	4,5	11,8	0,3	2	31	0,2
		Mid-adolescent	0	12	3,3	14,5	0,3	1	38	0,1
		Late-adolescent	0	13	2,0	14,9	0,2	2	44	0,2
		Adult	1	10	2,6	20,8	0,2	3	118	0,4
	Males	Early-adolescent	0	9	5,5	13,1	0,6	1	31	0,2
		Mid-adolescent	0	11	2,6	17,9	0,3	3	38	0,4
		Late-adolescent	0	12	5,4	18,8	0,5	1	43	0,1
		Adult	2	12	7,5	25,5	0,6	6	112	0,7
Palatable food	Females	Early-adolescent	0	14	3,8	11,9	0,3	2	31	0,2
		Mid-adolescent	1	14	2,5	14,6	0,2	0	38	0,0
		Late-adolescent	0	14	4,1	16,3	0,3	2	44	0,2
		Adult	2	10	4,6	20,7	0,4	2	115	0,3

Males	Early-adolescent	2	12	7,7	13,6	0,7	2	31	0,2
	Mid-adolescent	0	15	4,9	16,4	0,4	0	38	0,0
	Late-adolescent	0	15	2,8	18,9	0,2	1	45	0,1
	Adult	0	12	6,2	28,1	0,6	1	102	0,1

[a]Number of mice that did not meet criterion of spending less than 70% of time in any of conditioning contexts

Supplementary Table 2 Nutritional comparison of ingredients of palatable food mix used in food CPP test.

Component of palatable food mix	kcal/g	Carbohydrate %	Fat %	Protein %	Added Sugar %	Sodium %
Chow (Altromin)	3,3	65,0	11,0	24,0	-	0,2
Froot Loops	3,9	87,0	3,8	5,1	31,0	1,4
Cheetos	4,8	62,0	23,0	6,1	7,6	3,2
Oreos	4,8	68,0	20,0	5,3	38,0	0,7