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Socio-emotional behaviour in a VTA-shSHANK3 mouse model of ASD

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Summary

Abstract

Social deficits and stereotyped repetitive behaviours are the two core features of autistic spectrum disorders (ASDs) and animal models of these disorders can prove as valuable tools to investigate their mechanisms and causes. Here, we aimed first to better characterize rewarding aspects of social interaction in a three chambers task. Then, we developed a shorter protocol to perform social conditioning place preference (sCPP) and tested the requirement of the conditioning sessions to promote a sCPP by using a group of mice that underwent a trial where the contingencies were not kept between associations of stimuli and chamber. As social interaction can be aversive too, we developed the first protocol of social aversion place preference (sCPA) using an aggressive social stimulus. Finally, we tested the two core domains of ASDs and anxiety that is often comorbid in these disorders in a mouse model (shShank3) in which SHANK3 was downregulated specifically in the ventral tegmental area (VTA) that plays a key role in social motivation. Even thought a bidirectional social interaction is not required to promote a social preference it seems to be crucial to sustain and improve a reciprocal motivation, which will facilitate a stable long-term relationship. Our sCPP and sCPA protocols showed the relevance of the conditioning sessions and are time sparing. ShShank3 mice displayed social interaction deficits and repetitive behaviour but did not show any increased anxiety compared with controls.

Key words: ASDs, VTA, SHANK3.

Introduction

Autism Spectrum Disorders (ASDs) through history

The concept of infantile autism was first described in 1943 by American psychiatrist Leo Kanner, who characterized eleven children afflicted by affective and communication deficits without apparent cognitive disorder. One year later, Hans Asperger used the term "autistic psychopathy" to describe children with aberrant behavior in that, they had an elaborated, precocious but non-functional language and a broad range of intelligence. He postulated that autism has an organic origin and a genetic component, since the parents of the patients also displayed autistic features. However, Bruno Bettelheim, in 1950, formulated an alternative psychodynamic hypothesis, opposing the genetic one. According to him, the behavior of autistic children could be attributed to the detached behavior of the parents towards them and particularly to an unconscious idea from the part of the mother in that everything would be better if the child would not exist. Indeed, Bettelheim claimed to have cured 80% of those children separating them from their parents and taking care of them in a protected environment. In contrast, during the 70's, many family history-based and twin studies showed a 90% heritability of ASD traits, while not excluding environmental factors (1). Concordance rate of either autism or mild cognitive and social impairment was reported at a percentage of 82% for monozygotic and 10% for dizygotic twins while the recurrence rate in siblings was found to be approximately 20% (2). Moreover, earlier studies highlighted a marked increase in subclinical cognitive or behavioural deficits among family members with affected people compared to controls (3). During the decade of 80's, exon sequencing, next generation sequencing and genome wide association studies (GWAS), deepened the understanding of the genetic aetiology of ASDs (4). In addition, 5-15% of ASD individuals have a common known aetiology of ASD, like fragile X syndrome, tuberous sclerosis, trisomy 21 or Turner syndrome. However, about 5 % have a copy number variation and 5% have rare mutations in gene, such as *SHANK*. This suggests that ASD cannot be necessarily ascribed to one specific genetic alteration. Probably several genetic dysfunctions, such as mutations, copy number variations (CNV) and single nucleotide polymorphisms (SNPs) determine the appearance of the disorder. Additionally, some CNV studies in ASD patients identified mutations in genes also involved in other neuropsychiatric disorders, such as intellectual disability, schizophrenia and attention-deficit hyperactivity disorder, indicating that overlapping genetic alterations might lead to phenotypically distinct outcomes (5).

Since most of the candidate genes related to ASDs play a key role in synaptic functions, ASDs are often considered to be synaptic disorders or "synaptopathies" (6)(7).

The first diagnosis criteria of ASDs were described in the Diagnostic and Statistical Manual for Mental Disorder (DSM-III) but were quickly deemed as too restrictive. In 1994, being aware of the

huge heterogeneity of genetic disorders that could lead to a wide spectrum of clinical symptoms related to autism, the concept of pervasive developmental disorder (PDD) was progressively introduced owing to the DSM-IV. This term encompassed autism, Asperger syndrome, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified. The differentiation of one from the other was done using some special clinical features (3). Only a subset of symptoms was needed to form the diagnosis, increasing the sensitivity of detection and thus the prevalence. Since 2013, the DSM-V changed this segregated conception of PDD. Instead of having four different diagnostic domains, only one is now accepted: autistic spectrum disorder (ASD). For instance, Asperger syndrome is thought to be part of ASDs but is not considered as a separate disorder anymore (8). This new version of the DSM provides a classification based on the severity of deficits of two core symptoms characterizing ASDs: social communication and interaction deficits as well as restricted interests and repetitive behavior. These changes in diagnostic methodology allow a personalized assessment of each individual, improving the quality of the healthcare provided as well as their social integration. This conception of the autism as a spectrum of disorders, better reflects the current knowledge of this syndrome and should improve the diagnosis by clinicians without modifying negatively the sensitivity, but increasing the specificity of identification. A study performed in 2012 showed that the DSM-V criteria identified up to 91% of children that were diagnosed according to the criteria of the DSM-IV for PDD diagnoses. As a result, the prevalence of that disorder does not seem to change significantly between the last two versions of the DSM (9). Importantly, the new DSM-V diagnosis criteria, instead of being focused on school-age children like the previous version, encourage earlier patient identification and care (10). In order to be diagnosed as autistic, children have to show deficits in the two-abovementioned core domains since their early childhood, even though the problems are more evident later, when the social requirements exceed their ability to behave optimally. As described before, the first core symptom that was described in the DSM-IV is a persistent impairment in communication and social interactions. The child has difficulties in decoding emotions and sharing them with his/her relatives or shows incapacity to have a normal conversation with his peers. Additionally, he may have problems to adapt himself/herself in particular social situations because he cannot fully comprehend them. Repetitive behaviour, stereotyped actions and restricted interests constitute the second core symptom or domain. (11). Since several decades, ASD's prevalence has constantly been rising, estimated 1 in 68 children aged 8 years (12). This is translated to a huge economic burden worldwide. Indeed, without discussing about the indirect and intangible costs, the medical costs exceed 4.1-6.1 times those of children without ASD and the non-medical behavioural interventions cost 40'000 to 60'000$ per child per year in the USA (13). Understanding the underlying causes of ASD would allow for developing specific and efficient treatments. Moreover, improving the diagnostic criteria and methodology would mean that the diagnosis could happen earlier in life and thus, this would lead to better prognosis.

The social motivation hypothesis

One of the ASD symptom domains concerns social behaviour dysfunction. One hypothesis that has been brought forward and that might explain these impairments is the concept of deficits in social motivation. This hypothesis postulates that social interaction, recapitulated by three concepts social orienting, social seeking and social maintaining, is rewarding. Infants preferentially direct their attention to social stimuli, rather than objects. Moreover, when children have to detect small modifications and changes, they are more efficient when these alterations concern faces rather than objects (14) and they prefer to observe face-like stimuli rather than inverted or scrambled faces (15). In addition to this preferential attention to social stimuli, humans also seek to communicate and form bonds and collaborations, as these are pleasurable to them. In particular, this rewarding effect of social interaction is demonstrated when children are given the choice to collaborate performing a task together or doing it alone, children consistently choose the collaboration (16). Moreover, humans have conscious and unconscious strategies to engage in relationships. Indeed, individuals try to be likeable and increase communication with pairs either through flattery, trying to elicit positive feelings to the receiver, (17) or through unconscious nonverbal mimicry, also called the chameleon effect (18). On the contrary, social isolation in humans activates the same brain circuitry recruited by physical pain (19), leading to negative psychological states such as depression (20). Social isolation has been shown to have the same percentage of mortality risk as those of smoking or alcohol drinking. (21). All these evidences are highlighting the importance and significance of social interactions for human well-being and health. As a consequence, extended social isolation promotes anthropomorphism (attribute to some object or phenomenon human features), enhances the perception of others' kindness and improves non-verbal mimicry (22)(23).

Whether social deficits in ASD are caused by impairment in the processing of social information (perception, encoding, storage and adaptation to social situations- social cognition) or by deficits in social motivation is still an open question. In any case, it is plausible that impairment in assigning positive value to social stimuli might result in aberrant social experiences. As a consequence, the process of learning about and from social information could be affected. In this regards, reduced social motivation might affect social cognition later in life (16).

ASD children pay more attention at the background settings than in the characters while observing social images and show preference for non-social rather than social sounds (24)(25), indicating social orienting deficits. Additionally, ASD patients are less interested in collaborative actions (26) and report less pleasure interacting in pairs than typically developing individuals (27), suggesting social reward processing dysfunctions. Finally, ASD children seem to have deficits in maintaining social interaction through flattering behaviour (28).

Social interaction and communication in mice

As humans, rodents are a social species and their social interactions can be measured by several experimental paradigms.

1) The three-chamber task to study social preference and social novelty preference. In this task, the experimental mouse can freely explore an open area divided in three chambers and to interact either with a mouse or with an empty enclosure. A mouse will spend more time exploring the social stimulus compared to the inanimate object (social preference). In the second part of the test the mouse can choose whether to interact with a familiar or unfamiliar (i.e. novel) mouse. Mice systematically exhibit preference exploring the novel mouse. This three-chamber social preference task is largely used to test social interaction in ASD rodent models (29).

2) The conditioned place preference (CPP) test is used to assess the rewarding properties of food, drugs of abuse, alcohol etc. It is based on the concept that during the conditioning sessions, a mouse learns to associate a specific context or cue (CS) with a rewarding experience (e.g. consumption of food, drug intake, sexual intercourse), which represents the unconditioned stimulus (US). Then, in the post-test session, when given the choice between exploring either the reward-paired compartment or the non-rewarded one, if the mouse found indeed that the US was rewarding and if its learning and memory abilities are intact, it should spent more time in the paired compartment.

A similar task has also been used to investigate the rewarding effects of social interactions, through incentive motivation of interacting with a conspecific. As before, the concept is that the experimental mouse is conditioned by alternating between a cued-paired chamber containing a conspecific mouse (US+), and another chamber, with different and distinct contextual cues, that contain no social stimulus (US-). At the post-test, the mouse displays preference for the social conditioned chamber. This pleasurable experience is processed and associated with the relevant place which then shapes the future behavior of the mouse (30). A team showed that wild squirrels displayed a sCPP, indicating that social conditioned place preference (sCCP) is not an artifact of domestication(31).

In the literature there are several methods used to assess sCPP, which differ in terms of conditioning sessions. For instance, in both rats and mice a conditioning period of eight days with one session per day is usually adopted (32)(33–35)(36). However, other shorter (3-4 days) or longer (10 days) protocols have been performed (37–39) (40). Recently, a team developed a sCPP protocol with 2 days of conditioning (41). However, they adopted a non-counterbalanced procedure, systematically isolating the experimental animals during the last conditioning session (41). Therefore, with this protocol it is difficult to conclude if the obtained place preference is because the mice preferred the social-paired side or because they developed an aversion for the isolation-associated compartment. Indeed, *Panksepp et al.* demonstrated that the sCPP is based on both cues signalling social interaction

availability and cues that predict isolation and that social isolation was critical to produce a sCPP (40). To avoid this confounding parameter, some studies used a randomized initial pairing so that the subjects beginning with social pairing was counterbalanced with those starting with social isolation (37,38,42). In addition, there are three different ways to allocate animals to the pairing compartment. One of them consists in assigning randomly the paired chamber regardless of the baseline preference assessed by a pre-test session. This is an unbiased procedure. Another possibility is to pair the initially non-preferred side with a social stimulus and then assess if the animals acquire preference for this side (32–34,36,39,42). The third version consists in assigning the rodents to the paired-side before the pre-test and taking into account the mean pre-test preference in order to re-attribute some animals to obtain a preference score equal to the chance level (i.e. no preference) (35). Furthermore, the way that the post-test performance is assessed is also variable between studies. Actually, there are two main different methods. The first compares the pre-test with the post-test mean preference for the paired compartment (39,41,42). The second compares the social-paired chamber with the unpaired one according to the time spent in each of them during the post-test session (33,35–38,40).

The number of social-paring sessions (35,42) as well as the level of social motivation (35,40) influence the sCPP. This might be the reason underlying the extended periods of social isolation, which increases the subjective value of social interaction, found in the majority of sCPP protocols (32,34,36,37,39,42). Additionally, tactile stimuli perception seem to be one of the most rewarding components in social interaction (32) and the sCPP requires reciprocal play behaviours (35)(39). However, concerning the duration of conditioning sessions there are some discrepancies about its impact on sCPP response (35,42). In contrast with the favouring factors, there is a negative correlation between the weight difference between experimental and stimulus mouse and sCPP response (42). As a result, in sCPP experiments mostly performed on rats, animals are usually weight-matched (32–34,39,42). Another parameter that has to be taken into account before performing this experiment is the choice of the animal strain. Even though A/J, DBA/2J strains and C57BL/6J strain from the sub-strain Jackson showed a sCPP, the BALB/cJ and C57BL/6N from the NIH sub-strain failed (36,40). In parallel, sex-matched animals, mostly males (32,33,35–37,42) have been used for sCPP in order to avoid confounding parameter such as the natural preference of opposite sex conspecifics, or hormone levels effects on performance. Furthermore, animals at various ages have been subjected to the sCPP. More specifically, two periods are systematically selected: adolescence (35,38,40,42) and adulthood (32–34,36,37,41). However, since adolescent male rats displayed a stronger social preference compared to the older mice and to females (43) adolescent male rodents are probably more suitable to perform sCPP. Finally, regarding the behavioral apparatus used to perform the CPP, either three (with one neutral central compartment serving as corridor) or two chambers were used. Additionally, while the majority differentiated the compartments with only visual and tactile parameters (33–37,40,41) a team complemented the contextual environment with scented objects (42). Even though more cues

distinguishing the two compartments may be helpful for creating associations with the US, the possibility that the sCPP magnitude correlates with the number of specific cues is not clear.

Anxiety, another comorbidity of autism

Besides the two core symptoms of ASD, about 40% of ASD children exhibit a comorbid anxiety disorder (44). Anxiety is a psychological state during which someone assigns an emotional valence to an ambiguous environmental stimulus that will direct an adapted behaviour (45). Anxiety represents a state, in which negative valence is assigned to a stimulus not necessarily dangerous. This is associated with a high-arousal state that can enable the body to rapidly react to a potentially threatening event.

Some of the key brain regions involved in anxiety processing are the amygdala, the bed nucleus of stria terminalis (BNST), the prefrontal cortex (PFC) and the ventral hypoccampus (vHPC) (46). These regions process and assign an emotional valence to an environmental stimulus through sensory inputs processing. Moreover, the ventral tegmental area (VTA) plays a role in mediating anxiety. In particular, activation of excitatory vBNST-VTA connections increases anxiety-like behaviour in mice. On the contrary, stimulation of the inhibitory vBNST-VTA connections decreases anxiety-like behaviour while promoting reward seeking. However, whether VTA dysfunction is associated to anxiety-traits expressed by ASD patients is still an open question.

There are several protocols to characterize anxiety-like traits in rodents (47). One widely used behavioral paradigm that is employed to assess anxiety is the elevated-plus maze (EPM). This apparatus consists of four elevated arms, two open and two closed, that are elevated above the ground. Bright and open areas are stressful for rodents, as they may be more exposed to environmental threats. Thus, mice usually spend more time in closed arms and avoid visiting the open arms. The performance of the mice can be video-tracked and automatically scored during this task, allowing us to quantify and compare anxiety-like behavior between groups of mice (48).

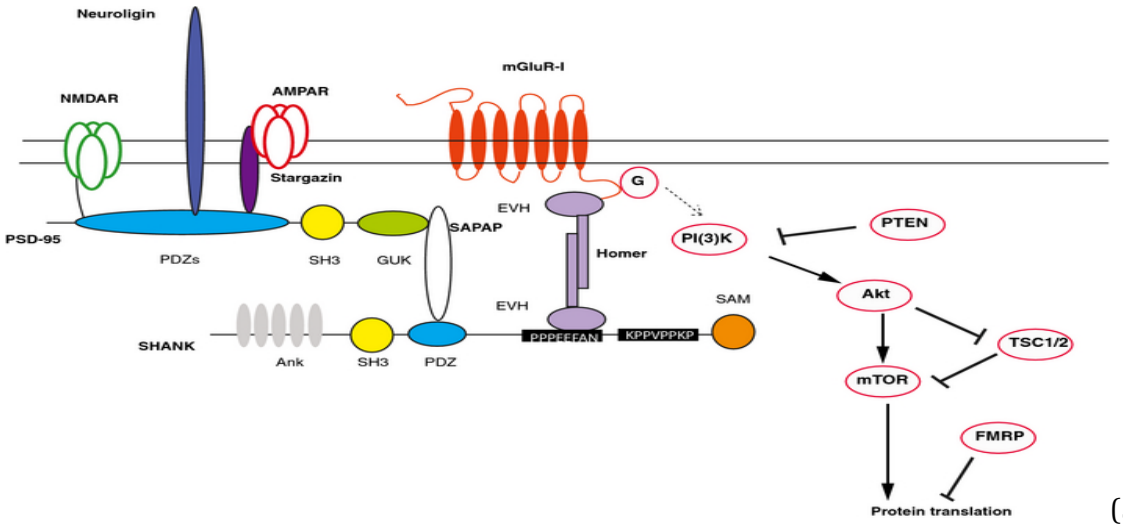
Phelan Mc Dermid syndrome (PMS)

Phelan McDermid syndrome is a rare neurodevelopmental disability that involves symptoms of the ASD spectrum. The symptomatology of PMS includes neonatal hypotonia, dysmorphic features, cognitive impairment, developmental delays with delayed speech (49). Comorbidities include reduced pain sensitivity, cardiac and renal malformations, recurring upper respiratory tract infections, gastroeosophageal reflux, lymphedema and strabismus. Interestingly, brain morphology alterations are found in more than 70% of affected patients. These malformations include thinning or hypoplasia of the corpus callosum, delayed myelination or global hypotrophy of the white matter, ventricular dilatation and cerebellar hypoplasia. Even if over than a thousand of penetrant genes yield to ASD, *SHANK3* haploinsufficiency (the lack of one copy of a gene) seems to be a prevalent and

underdiagnosed monogenic cause of ASD, explaining 0.5 % of cases. In comparison, fragile X syndrome and Rett Syndrome correspond respectively to about 2% and 0.5% of ASDs (49). The 75% of Phelan-McDermid patients presents *de novo* mutations or paternal loss of the distal segment of the long arm of the chromosome 22, more specifically, of the 22q13.3 region (50). Several studies have identified *SHANK3* as the most critical gene in that region because its selective deletion lead to PMS clinical features (51). Individuals carrying either a *de novo* frameshift or a non-sense mutation of *SHANK3*, show dysmorphic features, intellectual disability and autistic-like behaviour corresponding to the majority of the clinical features of Phelan Mcdermid syndrome (52). The same study even found a correlation between the severity of dysmorphic features, developmental delay and behavioural impairment severity and the deletion size of the gene.

Shank3 – structure and role in the post-synaptic density (PSD)

SHANK3, also known as PROSAP2, is a scaffolding postsynaptic protein enriched in the excitatory synapses. It is located in the postsynaptic density (PSD) which contains membrane proteins, scaffolding and anchoring proteins, signalling enzymes and cytoskeletal structures organized in a complex postsynaptic network (53). The full-length version of SHANK3 is composed by different domains: an ankyrin repeats domain (ANK), a Src homology 3 (SH3) domain, a PDZ domain that have a direct interaction with the GluA1-PDZ domain of alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid receptor (AMPAR), a prolin-rich region interacting indirectly with metabotropic glutamatergic receptor I (mGluR-I) through homer-protein, and a steril-alpha motiv (SAM) domain(54)(55).



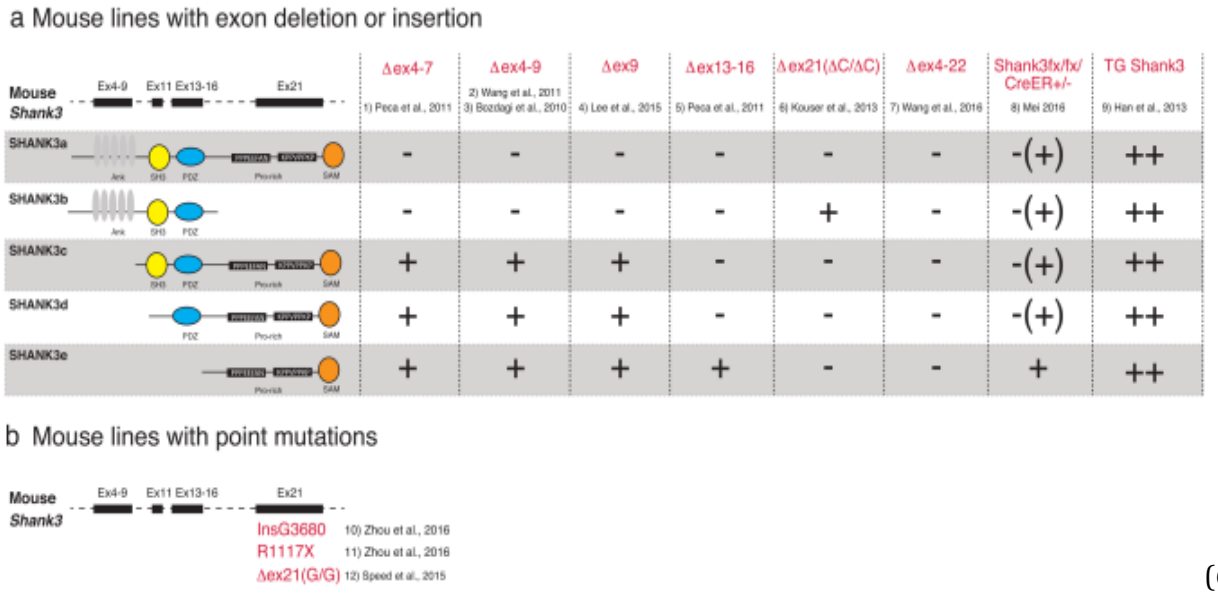
(54)

The protein complex PSD-95/SAPAP/SHANK3 orchestrates the assembly and signalling of the postsynaptic glutamatergic synapse (56). This protein network, bridging the group 1 mGluR (mGluR-I) and the ionotropic channels (iGluR; NMDARs and AMPARs), plays a crucial role in social interaction and emerges as a key point of convergence for genetic alterations in neurodevelopmental pathologies

(57)(56). In this regard, it has been shown that mGluR-I is necessary for the normal postsynaptic maturation of iGluRs in Ventral Tegmental Area (VTA) and might have a role in the maturation of other neural networks (58). In fact, during the first postnatal weeks, AMPAR transmission to VTA DA neurons is mainly supported by GluA2-lacking Ca++ permeable AMPARs whereas most NMDARs contain the GluN2B subunit. The activation of mGluR1 during the postnatal development promotes the removal of "immature" receptors, which are progressively replaced with GluA2-containing AMPARs and GluN2A-containing NMDA receptors.

In addition to synaptic maturation SHANK3 coordinates synaptogenesis and spine maturation. Indeed, over-expression of SHANK3 in cerebellar aspiny neurons is sufficient to induce dendritic spines and functional synapses formation (59). SHANK3 over-expression recruits glutamate receptors in PSD, increases the number/size of synaptic contacts and increases both the frequency and the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (59). Conversely, down-regulation of SHANK3 induces a reduction of mGLUR5 expression in the PSD and mEPSC frequency together with impaired mGLUR5-dependent synaptic plasticity (59). Treatment of hippocampal neurons lacking SHANK3 with 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)-benzamide, a positive allosteric modulator of mGLUR-5, restored mEPSC frequency *in vitro* (60).

Twelve transgenic mouse lines carrying various SHANK3 mutations have been developed to study its function from molecular, physiological and behavioural perspectives (figure below). There are several isoforms of this post-synaptic protein that are produced by several internal promoters (61). This results in a vast number of splicing variants and many different proteins (61) that have specific patterns of neuronal, regional and temporal expression (62).



(63)

Four lines of Shank3 knockout mice targeted the ANK repeats domain of SHANK3, inducing deletions in exons 4-9 (exon4-9J) (64) (exon 4-9B) (65), in exon 4-7 (Shank3a) (56) and in exon 9 (exon9) (66). As a consequence, there was a lack of the ANK-containing major isoforms of SHANK3

without disturbing the transcription of those starting further in the gene. Regarding the behavioral phenotype, except for the exon 9 model (66), these lines displayed impaired social interactions (64)(65)(56) as well as aberrant ultrasonic vocalisations (64) associated to social communication in rodents (67). Moreover, exon9 line displayed an increase in rearing (66), which could reflect increased anxiety-like behaviour, another ASD-related symptom. On the basis of self-grooming level, the exon 4-9J line showed an increase in repetitive behaviour, one of the core symptoms of ASD (64). Interestingly, although these *SHANK3* haplo-insufficiency models are similar, they display heterogeneous sociability impairments. This could be explained by different knockout strategies used. For example, exon 4-9 models lack a larger proportion of ANK repeats domain compared to the Shank3a and exon9 ones, which could explain the more severe social impairment in exon4-9 mouse line. Alternatively, differences in behavioural tasks or age at which animals were tested might also play a role.

A mouse model lacking the PDZ domain of SHANK3 displays impaired social exploration and preference as well as social recognition (56) together with increased self-grooming (56). Thirdly, in order to develop a model lacking all the major Shank3 isoforms, including the homer-binding-site ones, a research team produced a homozygote deletion of exon 21 in SHANK3 (68). Unlike other Shank3 transgenic models, these mutants expressed social preference during the three-chamber task (68). However, these mice showed impairment in social novelty together with an age-dependent increase in self-grooming, pointing to a time-/maturation-dependent phenotype expression of *SHANK3* haplo-insufficiency. In this regard, insertion of guanine nucleotide in exon 21, which creates a STOP-codon, resulted in the loss of the highest Shank3 molecular weight isoforms (69). In contrast with most of the aforementioned Shank3 mutant mice models, these mice neither displayed a significant impairment in social interaction nor an increase in repetitive and anxiety-like behaviours (69).

Finally, a complete Shank3 KO mouse model has been recently generated by deleting exons 4 to 22 resulting in a complete loss of all known SHANK3 mRNAs (70). Importantly, since in this model SHANK3 is completely removed and the most common mutation found in Phelan McDermid patients is the whole deletion on SHANK3 gene, this mouse line could represent a particularly suitable model for investigating PMS and SHANK3-related human ASD. Homozygous mice displayed all major SHANK3-related ASD features including increased repetitive behaviour, impaired communication and anxiety-like traits (70). Interestingly, the mice did not show a reduced social interest. However, homozygotes showed an increase in the non-reciprocated interaction with another mouse, suggesting that they may have impairment in interpreting social signals emitted by a conspecific. Actually, they showed a severe difficulty to engage in a reciprocal social interaction as the mouse they engaged systematically disengaged by ignoring and turning away from them. In the context of the social interaction test, homozygote mice increased their self-grooming behavior, suggesting repetitive behavior (70).

Despite some behavioural discrepancies in the previously described models, that are likely due to differential disruptions of isoforms and/or experimental methods, mutants carrying SHANK3 mutations represent a powerful tool to investigate the pathophysiology underlying ASD. As further described, new models of circuit-specific SHANK3 downregulation have been emerging and will continue to provide a better understanding of SHANK3 function in various neural circuits of interest and hopefully generate some new ASD treatment possibilities.

From social network to rewarding circuitry and dopamine signalling

Since 3-4 decades, the conceptualization of a social circuit has progressively been established and it comprises several brain regions such as Nucleus accumbens (NAc), basolateral-amygdala (BLA), pre-frontal cortex (mPFC), ventral hippocampus (vHPC) and ventral tegmental area (VTA). The NAc is a brain region involved in the initiation of reward-related behaviours and integrates inputs from cortical regions, such as mPFC and BLA, and subcortical regions, such as the VTA. While the prefrontal cortex is involved in the hedonic conscious representations (71), in emotion recognition and in interpersonal maintaining behaviours (72), the amygdala regulates behavioural responses according to the socio-environmental context by modulating the function of brain areas involved in social cognition (73). The VTA, located in the midbrain is constituted by dopamine (DA)-, GABA- and glutamate-releasing neurons. Since VTA DA neurons are responsive to either stimuli with a positive or negative valence, they modulate adaptive behaviour according to the motivational aspects of the experience (74)(75).

VTA DA neurons display a tonic low-frequency pattern of activity (76) that increases in response to environmental challenges. (75). In fact, an unpredicted rewarding stimulus induces a phasic firing of DA neurons (positive-prediction error). Conversely, while a total predicted reward elicits no change in the response of DA cells, a reward omission produces inhibition (negative-prediction error). Therefore, reward-predictive errors driven by DA neurons response could be resumed as reward prediction subtracted by reward occurred according to both magnitude and time-occurrence of the rewarding experience (77). Physiologically, an unexpected stimulus drives a prediction-error signal if a mismatch is detected between previous experiences, associated to the predictive reward, and the current stimulus. This signal may act as a trigger for downwards synapses modification resulting in a adaptation of the reward prediction as well as the behaviour associated to that stimulus (77). However, recently, it has been shown that DA neurons respond to aversive stimuli too. In fact, while the dorsal subpopulation displays inhibition in response to an aversive stimulus, the ventral one showed a phasic excitation (75). These findings suggest that two distinct VTA DA neuron populations either codes for rewarding or aversive experiences. This led subsequently to the hypothesis that distinct VTA dopamine neurons subpopulations target specific brain regions according to the valence of the stimulus. Several studies confirmed by retro-gradely labelled neurons that there

were distinct VTA DA cell's populations, each projecting to separate cerebral areas (78). Anatomically, there are two main brain target regions of VTA projecting neurons: medial prefrontal cortex and nucleus accumbens (79). While VTA-PFC pathway process aversive stimuli and seem to allow a behavioural flexibility (76), NAc projecting neurons are activated by rewarding experiences (78).

VTA and social behaviour

To understand the role of VTA DA neuron activity during social behaviour, a study reported activity changes in VTA DA neuron activity by measuring Ca^{++} transient changes in response to dyadic social interaction. During both social and novel object interactions, VTA neuron activity displays similar amplitude peak activity and decay across interaction time intervals. However, while for social interaction the peak activity corresponds to approaching or investigation behaviour, the one for object interaction is time-locked to withdrawal behaviour. Moreover, VTA peak activity predicts the latency to a next social interaction, the bigger the peak the shorter the latency to initiate a second interaction. Accordingly, optogenetic stimulation of VTA DA neurons (DAT-CRE mice injected by an AAV coding channelrhodopsin) increased social interaction while inhibition reduced it. Therefore, VTA DA neurons are both sufficient and necessary for social behaviour. However modulation of VTA activity did not affect object interaction. This differential modulation of exploratory behaviour supports the hypothesis of distinct VTA DA neuron subpopulations. While the PFC-projecting DA neuron stimulation was associated to aversive conditioning, the Ca^{++} concentration indirectly measured by fiber photometry showed a significant increase in VTA-Nac projection. Finally, to elucidate the postsynaptic transmission of this specific social circuit, through several mouse models involving optogenetics, they highlighted the sufficiency and necessity of NAc MSN D1R activation to induce social interaction (80).

Social interaction occurs naturally among mice and healthy humans, whereas ASD subjects seem to fail assigning value to social stimuli, resulting in social dysfunction (16). However, whether *SHANK3* haploinsufficiency found in phelan-McDermid patients induces social deficits through dysfunctions in specific brain regions, in particular the VTA, is an open question. Considering the role of VTA in modulating social behaviour and the involvement of SHANK3 in synaptic transmission and maturation, it would be therefore interesting to study whether SHANK3 downregulation restricted to the VTA would lead to social behaviour dysfunctions. A very recent study found that SHANK3 is involved in maturation of social reward circuit in VTA (81). Mice were injected in VTA before P6 by an AAV expressing shRNA targeting the prolin-rich region of SHANK3 mRNA, which resulted in a significant reduction SHANK3 expression compared to scrShank3 or uninfected mice. VTA DA neurons of shShank3 adolescent mice showed an increase in AMPA/NMDA ratio and in the rectification index suggesting the presence of GluA2-lacking AMPAR. While neonatal SHANK3 downregulation was leading to increase in both AMPA/NMDA ratio and RI, SHANK3 reduction starting from adolescence

did not, indicating that SHANK3 is required for the maturation of excitatory synapses of VTA DA neurons. Moreover, the firing rate of VTA DA neurons was significantly reduced. Conversely, VTA GABA neurons in shShank3 mouse showed a higher rate of firing but their RI was not modified. Therefore, the different deficits found in VTA DA and GABA neurons suggest a cell type-specific role of SHANK3. In parallel to synaptic and activity deficits, shShank3 mice showed a reduced social preference as well as an increase in self-grooming (81). Therefore, in addition to social behaviour deficits, VTA impairment seems to be involved in repetitive behaviour (82), the other ASD core symptom. That's why, among others, focusing on this brain region would be quite suitable to investigate some of the mechanisms underlying ASD.

Ultimately, neonatal treatment with a positive allosteric modulator of mGluR1 (PAM-mGluR1), which is required to drive synapse maturation in physiological conditions, was enough to normalize social preference as well as the general property of excitatory synapses and firing rate of VTA DA neurons. In particular, PAM-mGluR1 promoted a removal of GluA2-lacking AMPARs from shShank3 infected VTA DA neurons. Interestingly, even though the treatment lasted until early adolescence, social preference remained normal in adulthood. This paper confirms the involvement of VTA DA neurons in social behaviour (80) and highlights the crucial role of SHANK3 for the maturation of VTA DA neurons excitatory synapses (81).

Goals of the thesis

Social preference constitutes a rewarding experience for mammals which starts already early in life with play behavior between conspecifics (35,36). Social deficits is one of the core features of ASDs and animal models of these disorders can prove as valuable tools to investigate their mechanisms and causes.

One of the most widely used behavioral assay to study social interaction and preference in mice is the three-chamber test. However, the particular behavioral aspects involved in this task have been poorly characterized. The experimental animals in this task are exposed to two enclosures simultaneously. Typically, one enclosure contains another mouse and the other one is either empty or contains an inanimate object. However, no study has directly compared whether using an object or an empty enclosure would significantly affect the behavioral readout. Another question that rises from this paradigm is whether reciprocal interaction is required between the two mice (experimental and stimulus mouse) in order to obtain a social preference.

For this purpose, our first goal was to study in detail the abovementioned questions by designing experiments to explicitly address them. In condition 1, the experimental mice were exposed to an enclosure containing a mouse, as usual, or an empty enclosure, whereas in condition 2 the mice

where exposed to the enclosure with the mouse and to an enclosure that this time contained an object. In condition 3, we directly addressed the question of requirement or not of reciprocal social interaction by exposing the mice to the enclosures, one containing now an awake mouse as always while the other contains an anesthetized mouse. In condition 4, we asked the question whether the mice would prefer to interact with an anesthetized mouse or with an inanimate object, as we wanted to further explore what are the essential elements of social interaction that determine the development of social preference.

Although the assessment of behavior with the three-chamber test provides an indication of social approach behavior and preference when given a choice (for example between a social target and an object), the question whether social interaction is rewarding enough to promote associative learning, in a conditioned place preference paradigm, is not answered. This aspect of the rewarding properties of social interaction is really important when studying models of ASDs where we want to test the social motivation hypothesis. Additionally, the sCPP protocols described above are long and generally necessitate extended periods of social isolation, which can hamper the accuracy of studying the rewarding properties of social interaction. Our second goal was therefore, to develop a shorted sCPP protocol, based on repeated encounters with a young and unfamiliar conspecific, throughout the day and for 4 days.

One other aspect of social interaction can, in fact, be aversive. In human society bullying is an example of negative and stressful social interaction that constitutes an aversive experience to the victims. In order to fully understand the social behavior repertoire in mice of ASD models, it would be important to develop the tools to address the sensitivity of these mice to aversive social experience. Although there are protocols that assess conditioned place aversion (CPA) (83,84), to our knowledge nobody ever tried to perform this task with a social conditioning stimulus. Thus, our third goal was to develop and characterize a social conditioned place aversion (sCPA) protocol based on both concepts of the social defeat stress paradigm (85,86) and social conditioned place preference.

After having charecterized in detail the three-chamber social interaction task in goal 1, we selected one of the studied conditions and our fourth goal was to subject to this paradigm mice in which SHANK3 was downregulated selectively in the VTA as described above. Given that autism is often comorbid with elevated anxiety, one additional question was whether in the context of the social deficits displayed by shShank3 mice we would observe increased anxiety-like behavior. For this purpose, scrShank3 and shShank3 mice were subjected to the Elevated Plus Maze assay in order to explicitly quantify their anxiety-like behavior.

Materials and methods

Social interaction test

Animals: The experiment was conducted with 48 experimental and 24 stimuli C57Bl/6J mice. The age of the experimental animals was P60-P70 at the time of behavioral testing and both females and males were used in a balanced proportion. They were housed according to the sex in groups of 6 under normal light-dark cycle (lights on 7.00 am). Regarding the stimuli mice, 12 mice were utilized as the social stimulus and 12 of them were anesthetized during behavioral testing. All the behavioral experiments were performed during the light cycle. All the procedures performed at the UNIL and UNIGE complied with the Swiss National Institutional Guidelines on Animal experimentation and were approved by the Swiss Cantonal Veterinary Office Committee for Animal Experimentation. All procedures performed were conducted in accordance with the European directive 2010-63-EU and with approval from Bordeaux University Animal Care and Use Committee (no. 50120205-A).

Apparatus: A three-chambered social preference arena was used. It consisted of a rectangular Plexiglas arena (60×40×22cm) (Ugo Basile, Varese, Italy) divided into three chambers (each 20 × 40 × 22 (h) cm). The walls of the center chamber had doors that could be lifted to allow free access to all chambers (81).

Procedure: The preference for each test mouse is assessed according to the following conditions: social-object, social-empty, social-anesthetized and anesthetized-object. 12 mice were subjected in each of the above conditions. The social stimulus was a novel mouse that was used 3 times in total but 1 time per day. The object was a plastic yellow box of (3 x 2 x 7 cm). Regarding the anesthetized mouse, the anaesthesia was performed before the habituation period by a 50mg/kg dose intra-peritoneal injection of pentobarbital. This anesthetized mouse was used for two subsequent trials. The empty stimulus was the wire cage (enclosure) containing no object. The experiment took place over four days so that 12 test mice per day underwent the test. The stimuli (social, anesthetized mouse and object) were placed in a round enclosure, which allowed the test mouse to collect tactile, olfactory and auditory information without allowing other physical contact. The enclosures were placed in the two peripheral chambers before the habituation period during which the experimental mouse was placed in the central chamber and allowed to freely explore it for 10 minutes. Then, the doors were removed to allow the mouse to freely move in the entire arena for 10 minutes. To avoid any bias in place preference, in each condition, the position of the stimulus-containing enclosures was alternated and counterbalanced for each session. Between trials the entire arena was cleaned with a 5% ethanol solution. Every session was video-tracked and recorded using Ethovision XT (Noldus, Wageningen, the Netherlands), which provided an automated recording of several parameters such as the time spent around the enclosures, the entries, as well as the distance moved and the velocity. Other

behaviors such as sniffing, rearing and grooming were scored manually. The mice were considered to explore the stimuli when their nose was directed towards the enclosures' contents at a distance less than approximately 2 cm.

Stereotaxic injections: injections were performed as described by S. Briselli et al. (81). Basically, injection of purified AAV-shShank3 and AAV-scrShank3 were performed in mice at P5. The anaesthesia was induced and maintained with a mixture of Oxygen and Isoflurane (Baxter AG, Vienna, Austria). The animals were then placed on the stereotaxic frame (Angle One; Leica, Germany) and a single or bilateral craniotomy was made over the VTA at following stereotaxic coordinates: for neonatal injections (P2-P5) ML 0.15 mm, AP 0.1 mm, DV -3.8 mm from Lambda; for juvenile injections (P14/P21/P24) ML ±0.5 mm, AP -3.2 mm, DV -4.0 mm from Bregma. The virus was injected with graduated pipettes (Drummond Scientific Company, Broomall, PA) at the rate of 100 nl/min for a total volume of 50 and 200 nL for neonatal (as reported in the text) and 400 nL for juvenile animals. For all the experiments the virus was incubated for at least 9 days, when expression was clearly identifiable by the reporter protein expression, before proceeding with further manipulations. Every mouse was injected with either a vehicle-containing virus or a microRNA-containing one that targets the proline-rich domain of Shank3. As a result, shShank3 mice displayed a significant down-regulation of Shank3 protein in VTA of as compared to the scrShank3 ones. About 50% of VTA dopamine neurons were infected. The injection was specific to VTA since substantia nigra DA cells didn't show this reduction (81).

Social conditioned place preference (sCPP)

Animals: 8-14 weeks old C57Bl/6j male mice (body weight: 20-30g) were used for this test. 14 mice were always paired with the social stimulus in the same apparatus compartment that contains distinct contextual cues. The 9 remaining mice met the social stimulus in both compartments the same amount of time so that they couldn't associate one compartment with the rewarding social interaction. These experimental mice were housed in groups of 2. In addition, 3-week old C57Bl/6j male mice (body weight: 10-17g) where utilized as the social stimuli. They were housed alone after the pre-test session.

Apparatus: the Conditioned Place Preference (CPP) apparatus consists of 2 chambers connected by a corridor. One compartment has a smooth floor and grey lines on the walls, while the other has a rough floor and black dots on the wall (Bioseb).

Procedure: The experiment includes 3 phases over 6 days. At day 0, the pre-test session was performed, where the experimental mice freely explore the arena for 15 minutes. After the end of the pre-test session, the stimuli mice were habituated in their assigned chamber for 15 minutes each. On days 1-4, the conditioning sessions take place where the social stimulus (US) (i.e. stimulus mouse) was

either paired with one of the compartments (conditioning paradigm) or, for some animals, appears in both contexts in an alternating manner and for equal amount of time so that no association can be formed between context and stimulus (breaking the contingencies paradigm). Mice underwent a 30 min conditioning session once a day. The session was separated into six blocks of 5 min during which each mouse alternated between the two compartments, one containing the social stimulus (US+) and the other no stimulus (US-). We used the following schema: US+ (5min), US- (5min), US+ (5min), US- (5min), US+ (5min), US- (5min). Between each block the animal was guided through a corridor to the other compartment. At the end of each day's session, the animal was placed in its home cage and the entire apparatus was cleaned with a 1% acetic acid solution. Importantly, for each conditioning session, mice were counterbalanced across the days so that if one animal began the first day with a US+ session, it started with a US- session the following day. This design was followed in order to avoid any habitual action learning. In addition, the US+ sessions were counterbalanced on both sides, such that during the conditioning paradigm, half of the mice were conditioned with the social stimulus in the dotted compartment and half in the striped compartment. For the control experiment of breaking the contingencies, each mouse was conditioned with the social stimulus in alternating compartments. For example, during day 1 it interacted with the social stimulus in the dotted compartment and the second day in the striped one. Finally, during the 5th day mice underwent the post-test session, where they could freely explore the CPP apparatus for 15 minutes. The preference score is calculated as time spent in the social compartment / (time spent in the social compartment + time spent in the empty compartment).

Social Conditioned place aversion (sCPA):

For the CPA experiment 24 C57Bl/6J male mice were used as experimental mice and 13 CD1 male mice as stimuli mice (US+). In order to assess the aggressiveness of CD1 mice we put an intruder C57Bl/6 mouse in each CD1 cage for 5 minutes three consecutive days, where the latency of the first attack was measured. To be selected as the stimuli for the CPA, they had to attack with latency below 1 minute at least 2 consecutive days (87).

Apparatus: the same as for sCPP

The procedure of this experiment was similar to the CPP experiment described above except that the pre-test, the post-test and the conditioning sessions lasted 10 minutes. In addition, the conditioning sessions were separated into 10 blocks, each lasting one-minute. As in sCPP experiment, we used both paradigms (conditioning and breaking of the contingencies as a control experiment where no learning should occur).

Elevated plus maze (EPM)

Animals: 26 C57Bl/6J mice (8-10 weeks old) performed the experiment: 16 mice (9 females, 7 males) were injected with a virus downregulating Shank3 in the VTA (shShank3 mice) and 10 mice were injected with a virus that should not affect Shank3 expression (scrShank3) (9 males, 1 female). They were housed in groups of 2-3 according to sex and virus. 16 mice (9 Sh, 7 Scr) had done already a sucrose preference test before this experiment.

Procedure: the EPM was performed as described by V. Veenit et al. (88). Briefly, the test consists of two opposing open arms (50 × 10 cm) perpendicular to two enclosed arms (50 × 10 × 50 cm) that extend from a central platform (10 × 10 cm), elevated 65 cm above the floor. The mice were placed individually on the central platform facing a closed arm. They were allowed to explore the maze for 5 min. Their behaviour was monitored using a video camera and analysed with a computerized tracking system (Ethovision 3.1.16, Noldus IT, The Netherlands). The percent time spent and the number (frequency) of entries in the center, open and closed arms were recorded. Furthermore, in each arm, the "edge" was defined as the virtual zone corresponding to the last 10 cm of the arm. The entire apparatus was cleaned with 1% acetic acid solution and dried thoroughly between animals. The experiment was performed blindly to the experimental condition.

Results

Social preference does not require bidirectional social interaction in the social preference task

Our first goal was to better characterize the behavioral aspects of social interaction that are involved in the three chambers task. In the first condition, that is commonly used to address social preference in rodents, the experimental mouse had the choice of either exploring a confined mouse or an empty enclosure. As previously reported (28), the mouse spent more time sniffing its conspecific, suggesting its preference for social interactions (fig. 1a). Then, we attempting to change the saliency of the empty enclosure, an inanimate object was put inside (a yellow plastic rectangular box), such that the mice had to explore now either the enclosure containing a conspecific or the enclosure containing an object. In fact, the three chambers task is performed in many laboratories in this way, therefore, our question was whether using an enclosure containing an object or leaving it empty would affect social preference. In this context, the experimental mouse expressed a clear preference for the social stimulus, exploring the enclosure with the object for less time (Fig. 1b). Then, to address whether a reciprocal social interaction is required to induce social preference, the mice were given the choice to either explore an awake conspecific, as usual, or an anesthetized one. Surprisingly, they spent equal time sniffing both enclosures (fig. 1c). In a final experimental condition, we compared the time spent either with an anesthetized mouse or an object. The mice displayed an increased exploration of the anesthetized mouse (fig. 1d), suggesting that visual and olfactory parameters that are probably assimilated to social interaction sufficed to produce social preference. In order to compare all the experimental conditions between them, the preference score was calculated. As shown in figure 1e, mice displayed the same preference for a social stimulus regardless of whether an empty enclosure or an inanimate object was used and interestingly, regardless of the absence of reciprocal interactions with the social target. The preference score was equal when the animals had to explore either an awake or anesthetized mouse. Overall, our data suggest that reciprocal social interaction is not required to induce social preference and that an object-containing enclosure does not reduce the social preference. Finally, since self-grooming in mice can be considered either to reflect repetitive behavior, we wanted to assess this behaviour during all the abovementioned conditions. We observed that there was no significant change in the grooming level in any of the conditions studied (fig. 1f).

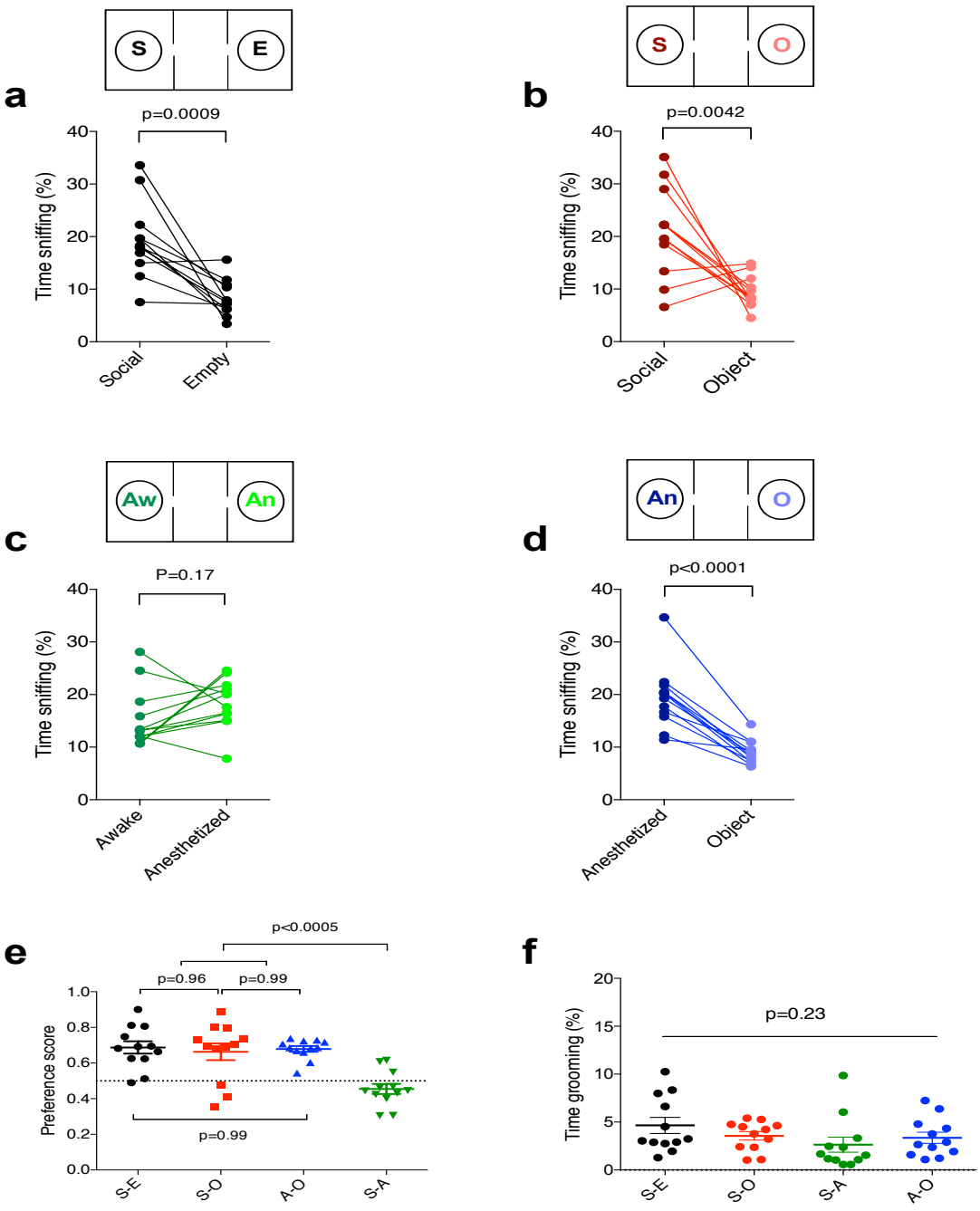


Figure 1

Figure 1. **Characterization of the three-chamber interaction task.** (a) Individual performance of mice for time sniffing the social target versus the empty enclosure ($t_{(11)}$ = 4.47, paired *t*-test). (b) Individual performance of mice for time sniffing the social target versus the object-containing enclosure ($t_{(11)}$ = 3.6, paired *t*-test). (c) Individual performance of mice for time sniffing an awake mouse versus an anesthetized one ($t_{(11)}$ = 1.47, paired *t*-test). (d) Individual performance of mice for time sniffing an anesthetized mouse versus the object-containing enclosure ($t_{(11)}$ = 7.73, paired *t*-test). (e) Scatter plots of preference score in each condition (one-way ANOVA F (3, 44) = 11.03, P < 0.0001, followed by tukey's multiple comparisons *post hoc* test). Preference score in S-E calculated by Time exploring social / (Time exploring social + time exploring empty). Results are the mean ± SEM. (f) Scatter plots of time grooming in each condition (one-way ANOVA F (3, 44) = 1.494, P = 0.23). Results are the mean ± SEM

In an attempt to study the temporal dynamics of social preference we divided the total experimental time in two equal time intervals (T1 and T2) and compared the preference score in T2 versus T1. While the social preference score remained steady in the social-empty condition (fig. 2a), it increased in the social-object paradigm (fig. 2b). In order to know whether this was due to an increase in the social or a decrease in the object interest, we compared both time points. While the time sniffing the social stimulus remained steady through time, mice spent less time sniffing the object in T2 (fig. 2c), suggesting a time-dependant loss of interest for the object-containing enclosure. In order to better understand the exploration dynamics in these two conditions, S-O ad S-E, we compared the empty and object exploration dynamics in these two paradigms. There was a non-significant decreased exploration in the S-O condition compared to the S-E, as the mean time sniffing the object-containing enclosure in T2 divided by T1 was not significantly lower than an empty enclosure itself (fig. 2d).

Then, even though the preference score for the anesthetized mouse in anesthetized-object showed a non-significant decrease (fig. 2e), the exploration time of the anesthetized mouse decreased in T2 and remained unchanged for the object (fig. 2f). Consistent with this analysis, mice lost their interest in the anesthetized mouse compared to the awake one, since time exploring the anesthetized mouse dropped in T2 (fig. 2g). We then sought to investigate the exploration temporal dynamics of a conspecific regardless of conscious state, and for this purpose we compared the anesthetized-object (A-O) and social-object (S-O) conditions by dividing the time exploration in T2 by the T1. This ratio was significantly lower for the anesthetized-object condition (fig. 2h), suggesting that although a reciprocal social interaction is not necessary to induce a preference, it seems to be critical to maintain the level of motivation across the time. Thus, our data suggest that although a social target seem to be enough to elicit preference over an inanimate object, regardless of reciprocity, there are, in fact, behavioural parameters concerning the temporal dynamics of exploration that delineate these conditions.

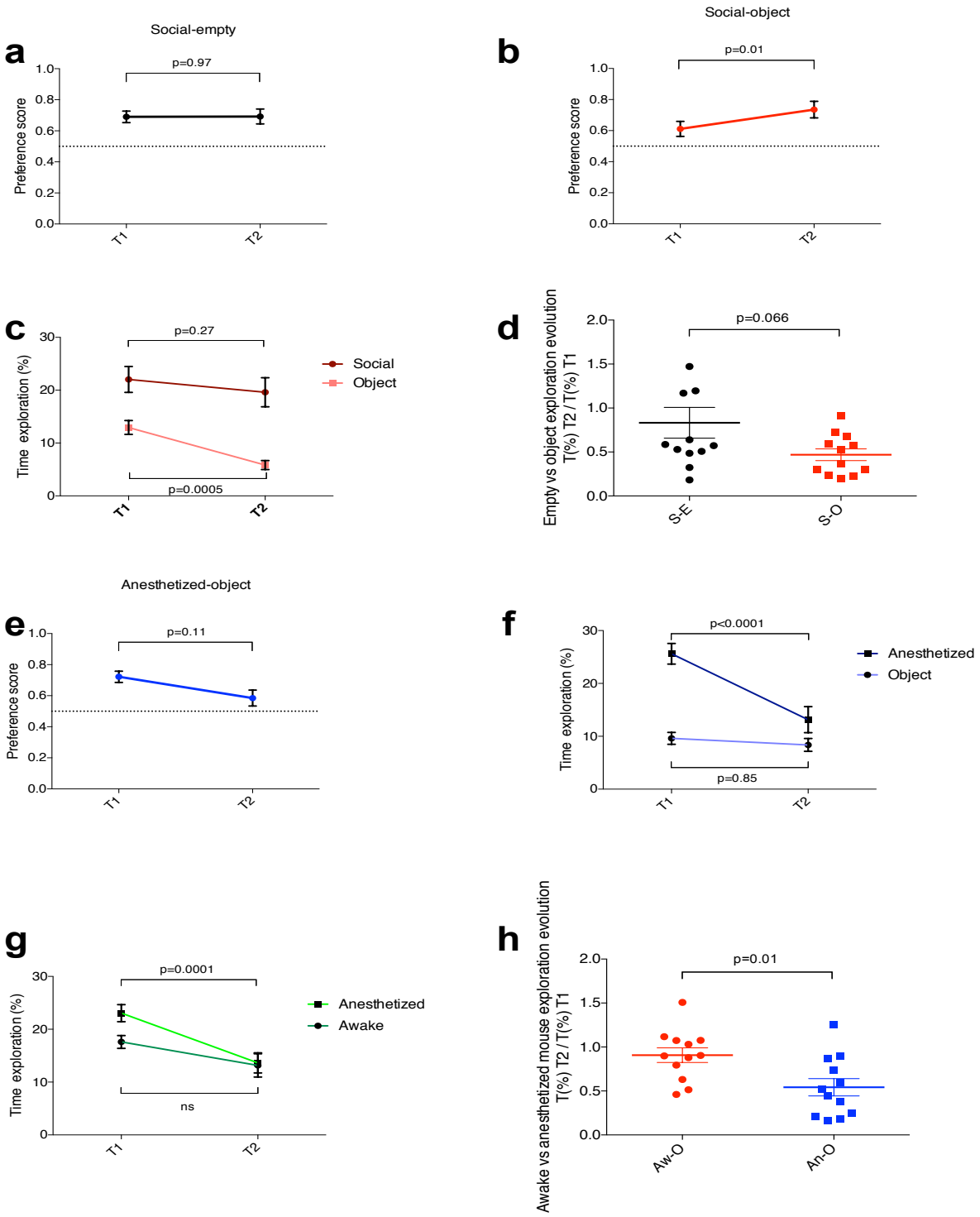


Figure 2

Figure 2. Exploration dynamics in three chambers task where T1 and T2 represent two 5-minute intervals. (a) Social preference score in T1 and T2 in social-empty condition ($t_{(11)}$ = 0.04, paired *t*-test). Social preference score is Time exploring social / (Time exploring social + time exploring empty). (b) Social preference score in T1 and T2 in social-object condition ($t_{(11)}$ = 3.09, paired *t*-test). (c) Time sniffing the social target versus the object-containing enclosure in T1 and T2 (two-way ANOVA ; time x group interaction : $F_{(1.22)}$ = 4.18, P = 0.053 ; main time effect : $F_{(1.22)}$ = 17.38, P = 0.0004 ; main group effect : $F_{(1.22)}$ = 19.55, P = 0.0002 ; followed by Sidak's multiple comparisons *post hoc* test). (d) Scatter plots of dynamics of time sniffing the empty enclosure in social-empty condition and the object-containing enclosure in social-object condition ($t_{(22)}$ = 1.93, unpaired *t*-test). Results are the mean (T(%) sniffing in T2 divided by T(%) sniffing in T1) ± SEM. (e) Anesthetized

preference score in T1 and T2 in anesthetized-object condition ($t_{(11)}$ = 1.72, paired *t*-test). (f) Time sniffing the anesthetized target versus the object-containing enclosure in T1 and T2 (two-way ANOVA ; time x group interaction : $F_{(1.22)}$ = 10.93, P = 0.032 ; main time effect : $F_{(1.22)}$ = 16.27, P = 0.0006 ; main group effect : $F_{(1.22)}$ = 31.89, P < 0.0001 ; followed by Sidak's multiple comparisons *post hoc* test). (g) Time sniffing the anesthetized target versus social target in T1 and T2 (two-way ANOVA ; time x group interaction : $F_{(1.22)}$ = 3.43, P = 0.078 ; main time effect : $F_{(1.22)}$ = 26.5, P < 0.0001 ; main group effect : $F_{(1.22)}$ = 1.97, P = 0.17 ; followed by Sidak's multiple comparisons *post hoc* test). (h) Scatter plots of dynamics of time sniffing the social target in social-object condition and the anesthetized target in anesthetized-object condition ($t_{(22)}$ = 2.82, unpaired *t*-test). Results are the mean (T(%) sniffing in T2 divided by T(%) sniffing in T1) ± SEM.

Development of a novel social Conditioned Place Preference task (sCPP)

According to our previous experiments, we found that bidirectional social interaction is critical to keep the motivation to engage in such relations. Although, the three chambers task is usually used to assess social preference, the question whether social interaction is rewarding enough to promote associative learning in a sCPP experiment remains open. Moreover, several protocols used to perform this task are often time consuming. Our second goal was therefore, to develop a shorted sCPP protocol, based on repeated encounters with a young and unfamiliar conspecific, throughout the day and for 4 days. Mice explored for more time the chamber associated with a social target during the conditioning sessions compared with the empty chamber (Fig. 3a), indicating that social contact reinforced the behaviour of the mice in that they learned and remembered the place that they experienced social interactions. Moreover, the time spent in the social-paired chamber raised in the post-test session (Fig. 3b). In order to further validate our protocol, we included a contingency break experiment where a second group of mice underwent the same experience, but now the social-paired side was inversed each day, thus not having a specific chamber associated with the social stimulus. Under this condition, no place preference was observed, since the time exploring both chambers was equal between the two chambers (context A and context B) (Fig. 3c) and between pre- and post-test (Fig. 3d). We then compared the pre-test and post-test preference scores by assessing the time spent in the paired-side / (the time spent in the paired-side + the time spent in the unpaired-side). As expected, mice from both "paired" and "contingency break" groups did not show any preference during the pre-test session as they explored about 50% of the time each chamber. However, during the post-test session we found a significant difference in the preference scores between both groups with an increased exploration of the social-paired chamber for the "pairing" group (Fig. 3e). Finally, we assessed whether this acquisition of preference was also reflected in the number of visits of both compartments, however, this was not the case (Fig. 3f).

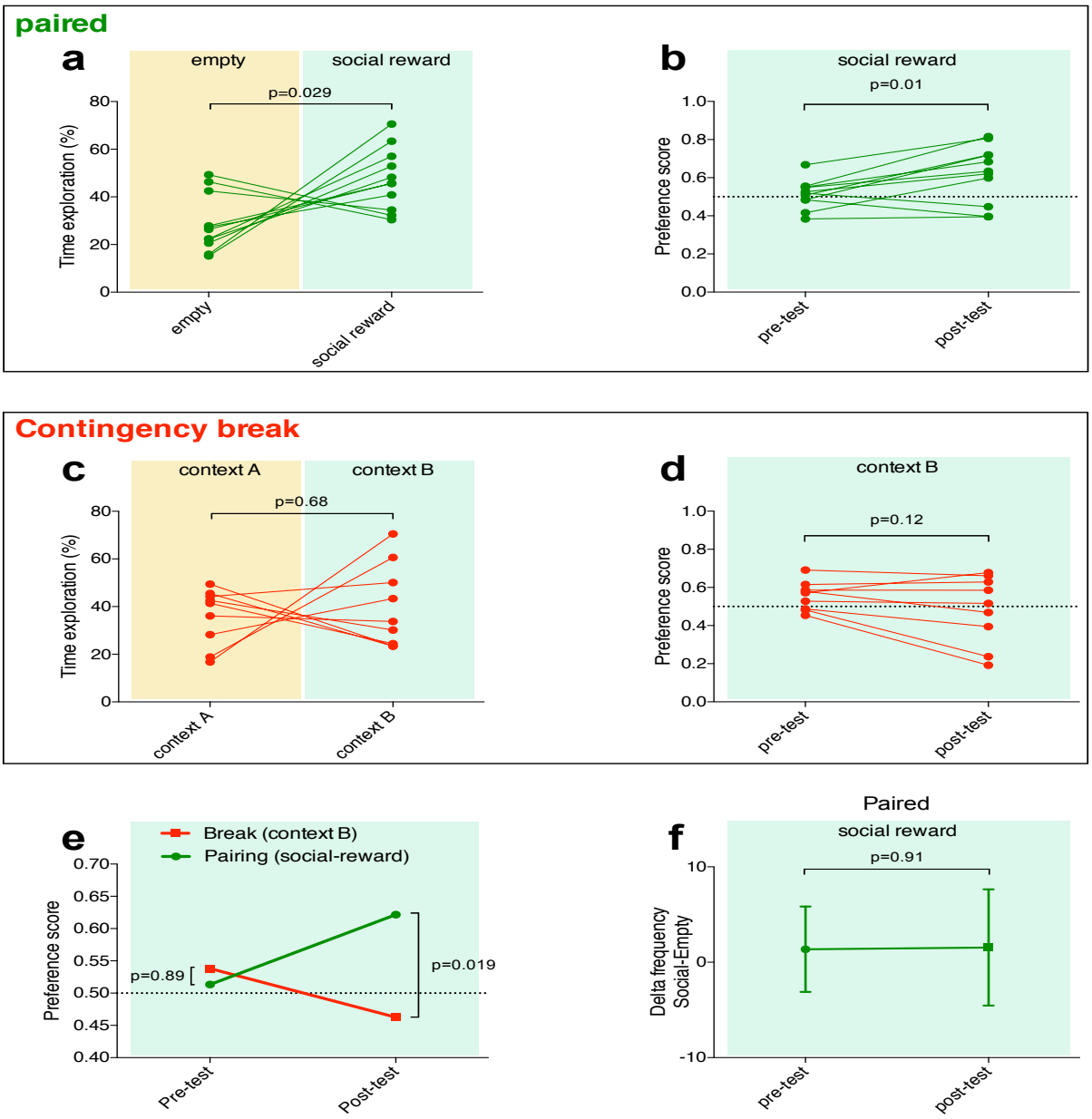


Figure 3

Figure 3. Social Conditioned Place Preference test. (a) Individual performance of mice in the pairing paradigm for time exploring the empty chamber versus the social-stimulus paired one in the post-test session ($t_{(10)}$ = 2.55, paired *t*-test). (b) Individual preference score of mice for the social-paired chamber in the pairing paradigm in pre- versus post-test sessions ($t_{(10)}$ = 3.07, paired *t*-test). (c) Individual performance of mice in the contingency break paradigm for time exploring the context A versus context B in the post-test session ($t_{(8)}$ = 0.43, paired *t*-test). (d) Individual preference score of mice in the contingency break paradigm in pre- versus post-test sessions ($t_{(8)}$ = 1.75, paired *t*-test). (e) Preference scores for the social-paired chamber of the paired paradigm group and for the context B chamber of contingency break paradigm in pre- versus post-test sessions (two-way ANOVA ; time x group interaction : $F_{(1.17)}$ = 10.54, P = 0.005 ; main time effect : $F_{(1.17)}$ = 0.22, P = 0.57 ; main group effect : $F_{(1.17)}$ = 1.76, P = 0.2 ; followed by Sidak's multiple comparisons *post hoc* test). (f) Difference of frequency (number of explorations of the social-paired chamber minus the number of exploration of the empty) in the paired condition ($t_{(10)}$ = 0.11, paired *t*-test). Preference scores are the Time exploring social / (Time exploring social + time exploring empty).

Development of a novel social Conditioned Place Aversion task (sCPA)

To our knowledge, there are no publications about conditioned place aversion with an aversive social stimulus. We therefore employed both concepts of social defeat stress and sCPP to develop a new social conditioned place aversion (sCPA) procedure. As in the sCPP experiment, a chamber was paired with a social stimulus that this time was an aggressive CD1 mouse and the other side was empty. Moreover, we sought to assess whether only 1-minute long conditioning sessions repeated ten times per day during four consecutive days would produce a sCPA. In fact, mice spent less time in the aggressor-paired chamber than in the empty one during the post-test session (Fig. 4a). Moreover, the preference score for the aggressor-paired compartment decreased between pre- and post-test sessions, suggesting that the experimental mice learned to associate the aggressor-paired chamber with an aversive experience and avoid it (Fig. 4b). Similarly to the sCPP, mice that were exposed to the same experience of defeat but the stimulus-chamber pairings were not consistent (Contingency Break condition) could not associate the chambers to the social aversive and they did not show any preference in the post-test session (Fig. 4c). As expected, they did not display any associative learning between pre- and post-test sessions (Fig. 4d). The comparison of the preference score of both groups (paired vs contingency break) in pre- and post-test session revealed that while in pre-test session the preference score was similar (about 50%) in the paired and contingency break conditions, this score differed significantly between the groups after the conditioning sessions (Fig. 4e). Finally, the comparison of the differential number of entries between both chambers, contrary to the sCPP experiment where no differences were observed, indicated a reduced number of entries in the chamber associated with a CD1 mouse in the post-test compared to the pre-test (Fig. 4f). This difference between the sCPP and sCPA experiments could suggest that the encounter with an aggressive mouse could be more salient in that it promotes associative learning. However, when we compared the preference score in the post-test session of sCPP and the aversion score of sCPA experiments we did not observe any difference between paradigms (Fig. 4g), suggesting that both aversive and rewarding experiences can be equally salient to promote learning and guide future behaviour

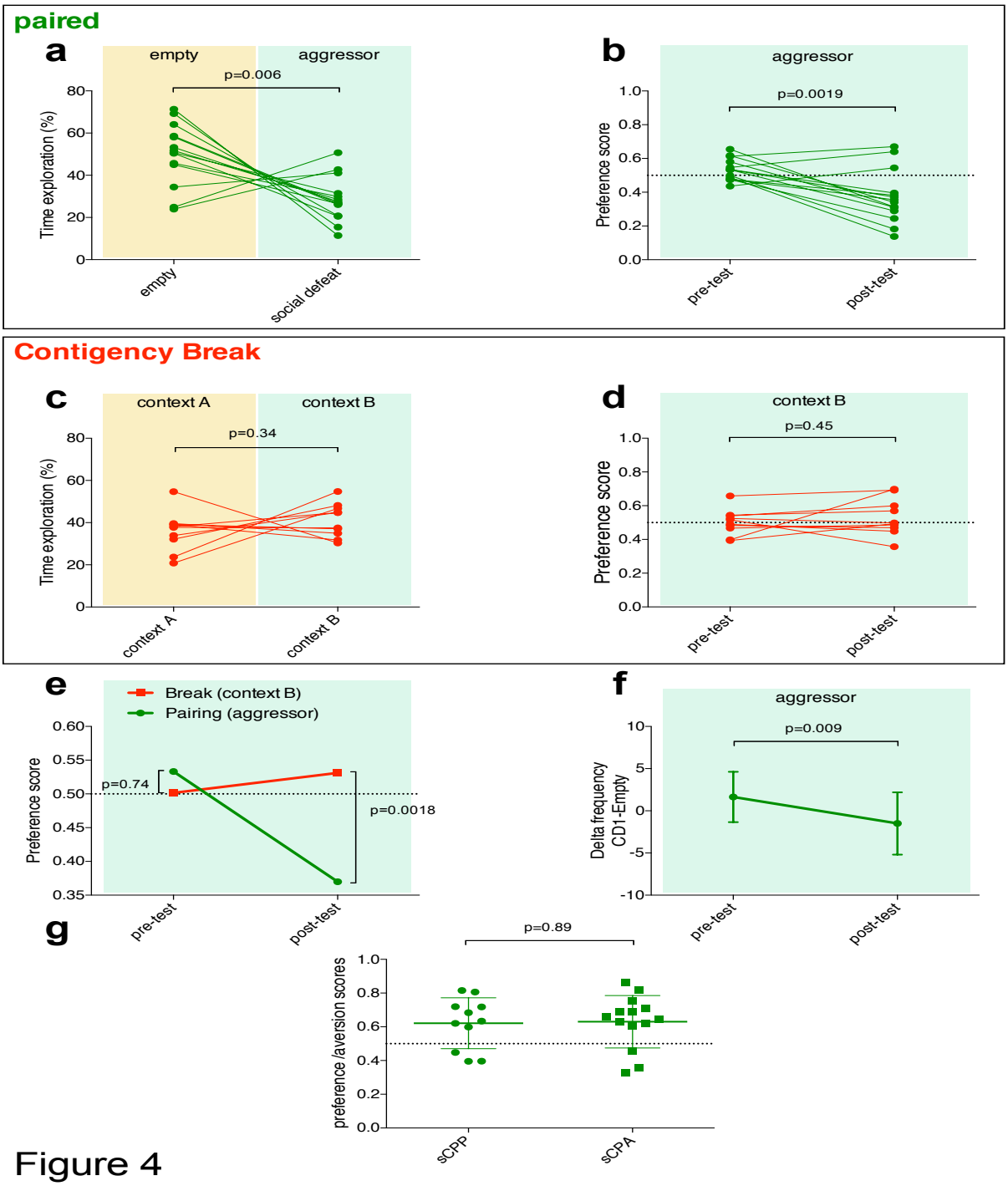


Figure 4

Figure 4. Social Conditioned Place Aversion test. (a) Individual performance of mice in paired paradigm for time exploring the empty versus the aggressor-paired chamber in the post-test session ($t_{(13)}$ = 3.26, paired *t*-test). (b) Individual preference score of mice for aggressor-paired chamber in paired paradigm in pre-test versus post-test sessions ($t_{(13)}$ = 3.89, paired *t*-test). (c) Individual performance of mice in contingency break condition for time exploring the context A chamber versus the context B in the post-test session ($t_{(9)}$ = 0.1, paired *t*-test). (d) Individual preference score of mice for the context B chamber in contingency break paradigm in pre-test versus post-test sessions ($t_{(9)}$ = 0.8, paired *t*-test). (e) Preference scores for the aggressor-paired chamber of the pairing group and for the context B chamber of the the contingency break group in pre-test versus post-test sessions (two-way ANOVA ; time x group interaction : $F_{(1.22)}$ = 10.72, P = 0.0035 ; main time effect : $F_{(1.22)}$ = 5.13, P = 0.03 ; main group effect : $F_{(1.22)}$ = 3.6, P = 0.07 ; followed by Sidak's multiple comparisons *post hoc* test). (f) Delta frequency for the pairing group in pre-test versus post-test sessions ($t_{(13)}$ = 3.1, paired *t*-test). Delta frequency is the number of explorations of the aggressor-paired chamber minus the number of exploration of the empty one. Preference scores are Time exploring aggressor / (Time exploring aggressor + time exploring empty). (g) Scatter plots of preference score versus aversion score in the sCPP and the sCPA experiments

respectively in the post-test session for the paired condition ($t_{(23)}$ = 1.14, unpaired *t*-test). Aversion score = 1 – preference score for aggressor-paired chamber.

VTA-SHANK3 downregulation induces social deficits and stereotyped repetitive behaviours

Several models of Shank3 KO mice exhibit social impairments as well as increased repetitive behaviours. Moreover, previous findings showed that the specific downregulation of SHANK3 in the VTA promotes social deficits (81). Our fourth goal was to use our conclusions from goal 1, that the social-empty variation of the three chambers task was an optimal condition for studying social preference, in order to assess the social preference of mice in which SHANK3 had been downregulated in the VTA (shShank3). First, we performed this task with mice (scrShank3), which were injected in the VTA as the shShank3 mice, but with a viral construct that does not interfere with SHANK3 expression (81). As in the abovementioned three chambers experiment, scrShank3 controls spent more time sniffing the social target than the empty enclosure (Fig. 5a). When we tested shShank3 however, we observed that they could not discriminate between the social stimulus and empty enclosures (Fig. 5b). Moreover, the comparison between both groups revealed a significant difference in the time sniffing the social target (Fig. 5c). These results confirm that, conversely to the control mice, shShank3 do not display any social preference. Further analyses showed that shShank3 mice displayed a time-dependant loss of interest for social interaction since the time sniffing the social target was significantly lower in the last five minutes interval (T2) than in T1. In contrast, scrShank3 mice showed a non-significant decrease of time exploring the social stimulus (Fig. 5d).

Since shShank3 mice displayed social impairments, the first core symptom of ASD, we wanted to assess whether VTA-*SHANK3* insufficiency would promote increased repetitive behaviour. Although, the grooming level of both groups was identical (Fig. 5e), as their social preference tend to decrease in T2, shShank3 mice exhibited a higher level of self-grooming than scrShank3 mice, suggesting a time-dependant increase of repetitive behaviour (Fig 5f).

Collectively, our data indicate that the specific downregulation of SHANK3 in the VTA promotes both core symptoms of ASDs, namely, impaired social interactions and increased repetitive behaviours.

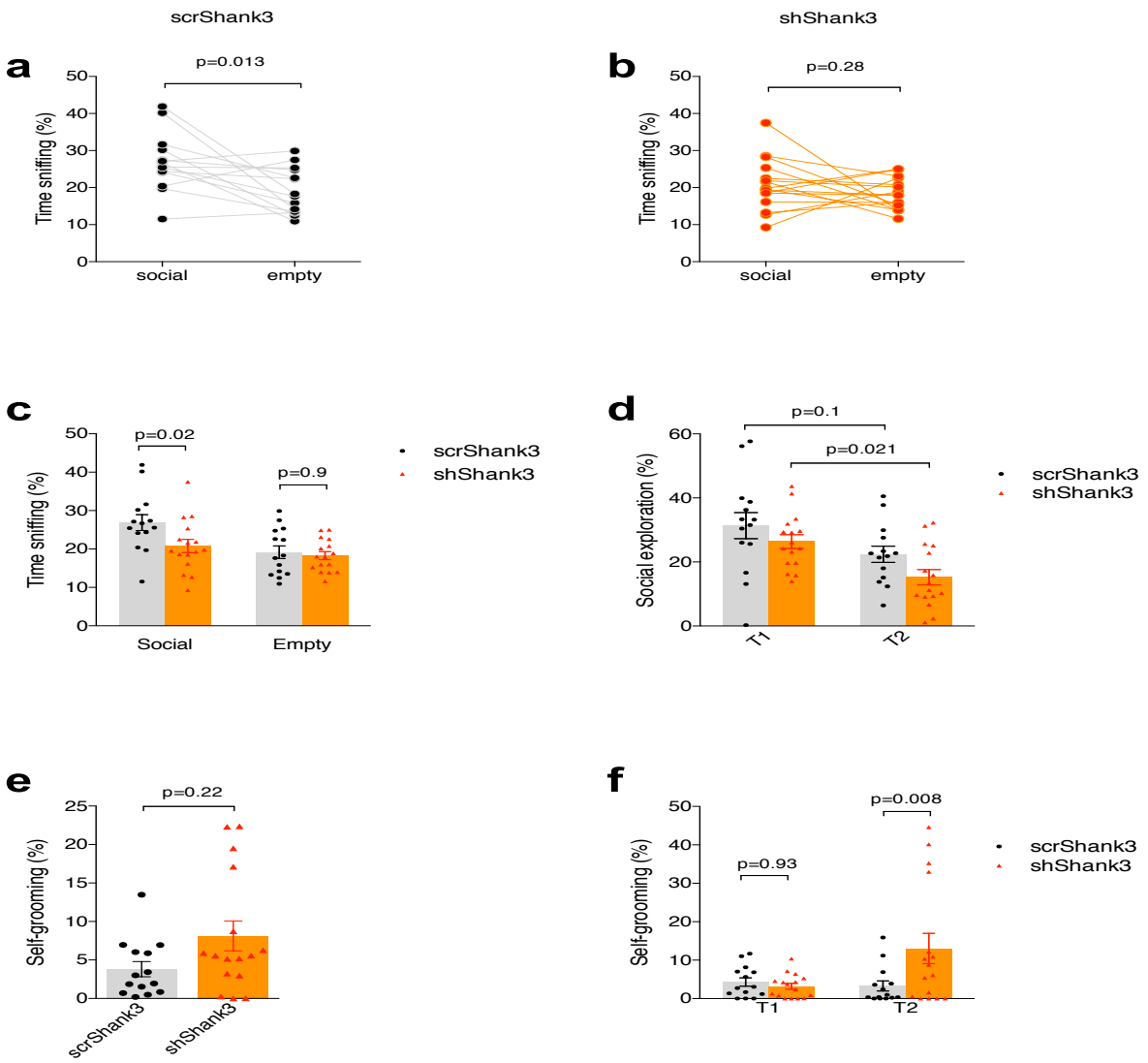


Figure 5

Figure 5. Three chambers test under the social-empty condition for scr- and shShank3 mice. (a) Individual performance of scrShank3 mice for time sniffing the social target versus the empty enclosure ($t_{(13)}$ = 2.89, paired *t*-test). (b) Individual performance of shShank3 mice for time sniffing the social target versus the empty enclosure ($t_{(15)}$ = 1.13, paired *t*-test). (c) Scatter plots and bar graphs of scrShank3 and shShank3 mice for time sniffing the social target versus the empty enclosure (two-way ANOVA ; stimulus x group interaction : $F_{(1.56)}$ = 2.46, P = 0.12 ; main stimulus effect : $F_{(1.56)}$ =9.73 , P = 0.003 ; main group effect : $F_{(1.56)}$ = 4.55, P = 0.004 ; followed by Sidak's multiple comparisons *post hoc* test). (d) Scatter plots and bar graphs representing the dynamics of social exploration for both groups (two-way ANOVA ; time x group interaction : $F_{(1.28)}$ = 0.13, P = 0.72 ; main time effect : $F_{(1.28)}$ = 11.35, P = 0.002 ; main group effect : $F_{(1.28)}$ = 5.16, P = 0.03 ; followed by Sidak's multiple comparisons *post hoc* test. (e) Scatter plots and bar graphs of scrShank3 and shShank3 for time self-grooming (U = 82, Mann-Whitney). (f) Scatter plots and bar graphs representing the dynamics of self-grooming for both groups (two-way ANOVA ; time x group interaction : $F_{(1.28)}$ = 5.47, P = 0.0027 ; main time effect : $F_{(1.28)}$ = 3.62 , P = 0.07 ; main group effect : $F_{(1.28)}$ = 3.65, P = 0.07 ; followed by Sidak's multiple comparisons *post hoc* test). Time is always expressed as the percent of the whole experiment period. T1 and T2 are two intervals of 5 minutes each. All results of scatter plots are the mean ± SEM.

VTA-SHANK3 downregulation does not induce anxiety-like behaviour

As previously reported, shShank3 mice displayed social interaction deficits and an increased level of repetitive behaviours. To further characterize this ASD-related mouse model, we wanted to assess whether shShank3 mice would show an increased level of anxiety-like behaviour that is often comorbid in ASDs by performing an elevated plus maze (EPM) task. In comparison to control mice, shShank3 did not show reduced exploration of the open arms (fig. 6a) or an increase of the closed arms, that would indicate augmented anxiety behaviour (fig. 6b). In order to be more specific, we measured the time spent in the open edges (10 cm of the open arms) that could represent the most stressful areas because of the increased exposure to environmental threats. However, shShank3 mice did not display an increased avoidance of these areas compared to control mice (figure not shown). In addition, the frequency of open edges exploration as well as the latency to the first open edge entry, both groups behaved similarly (fig. 6c-d). Finally, consistent with previous findings (81), shShank3 mice did not display any motor deficit on the basis of their mean velocity during the test compared to scrShank3 (fig. 6e). Together, these results suggest that, although shShank3 mice show impaired social interaction and repetitive behaviours, they did not display any increased anxiety-like behaviour. This suggests that even though the VTA plays a role in the modulation of anxiety (47), SHANK3 down-regulation in this specific region does not seem to increase anxiety levels as measured with the EPM.

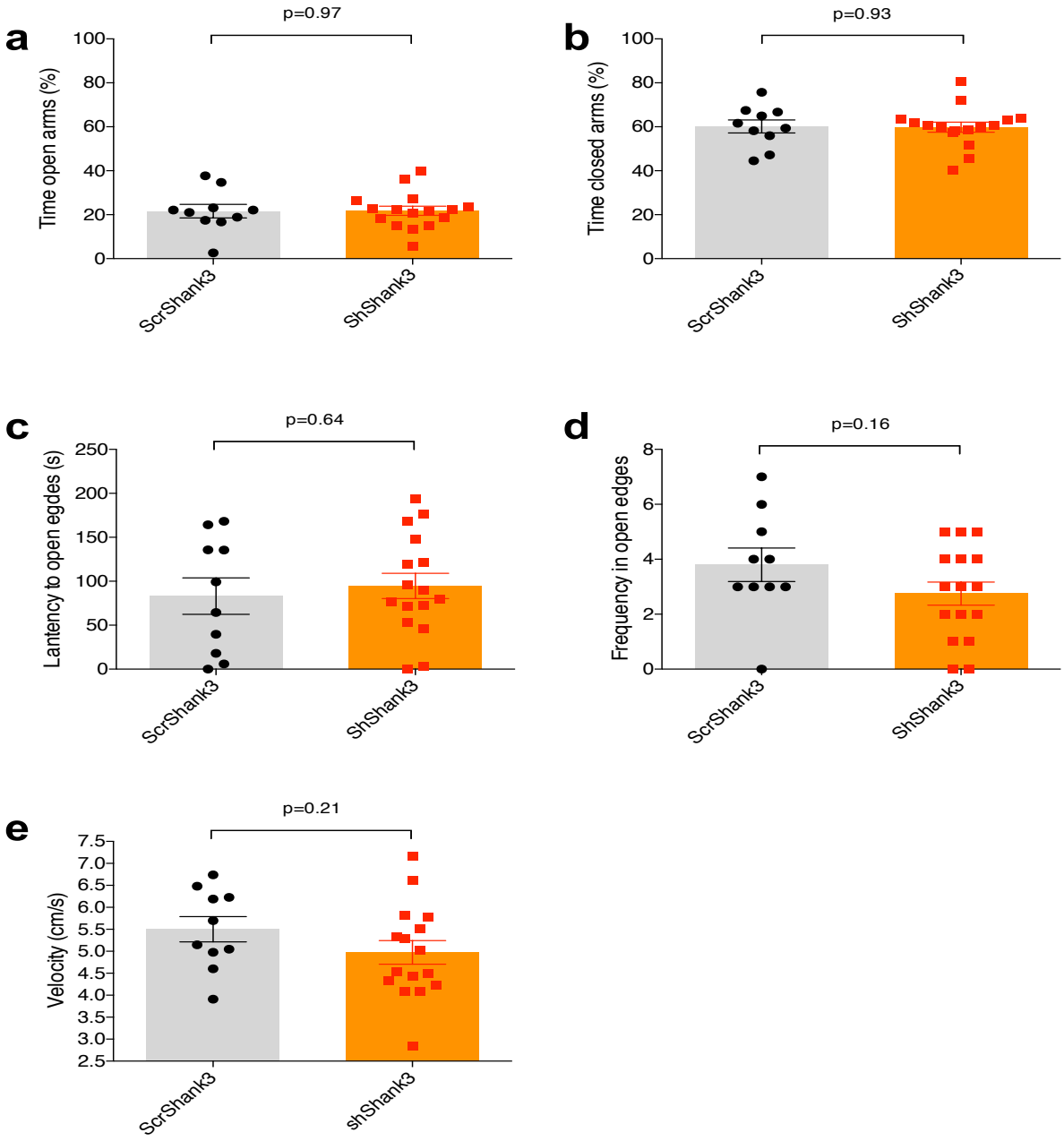


Figure 6

Figure 6. Characterization of Elevated Plus Maze task. (a) Scatter plots and bar graphs of individual performance of mice for time exploring the open arms ($t_{(24)}$ = 0.04, unpaired *t*-test). (b) Scatter plots and bar graphs of individual performance of mice for time exploring the closed arms ($t_{(24)}$ = 0.09, unpaired *t*-test). (c) Scatter plots and bar graphs of individual performance of mice for latency to explore the open edges ($t_{(24)}$ = 0.47, unpaired *t*-test). (d) Scatter plots and bar graphs of individual performance of mice for number of open edges entries ($t_{(24)}$ = 1.49, unpaired *t*-test). (e) Scatter plots and bar graphs of individual performance of mice for velocity ($t_{(24)}$ = 1.29, unpaired *t*-test). Time is expressed as percent of the whole experiment period. Results are the mean ± SEM.

Discussion

The pursuit of social interactions is a fundamental behavior of mammals with strong rewarding properties in which play behaviours during early life and tactile stimuli are crucial (32,35). We designed a paradigm in which bidirectional social interaction was abolished by anesthetizing the stimulus mouse and we were able to demonstrate that only olfactory, visual and tactile cues were sufficient to induce social preference. More specifically, mice showed the same magnitude of preference for either an awake or an anesthetized conspecific when the other choice was a yellow plastic rectangular box. In addition, they spent the same amount of time interacting with an awake and anesthetized mouse. This suggests that a bidirectional social interaction is not necessary in order to observe social preference. It is conceivable that some other clues of social interaction have sufficient rewarding properties to induce a social preference. Tactile, visual and olfactory cues of social interaction may act as predictors of bidirectional social interaction, which is rewarding, and so are sufficient to elicit social preference. However, taking into account temporal dynamic analysis, experimental mice showed a decreased exploring time when the conspecific was anesthetized. On the other hand, the exploring time remained stable when the choice was between an awake and an anesthetized stimulus mouse and the total duration of the task was taken into account. As a result, it seems that bidirectional social interaction may sustain and improve a reciprocal motivation, which will facilitate a stable long-term relationship. When we modified the saliency of the empty enclosure by putting inside an inanimate object, interestingly, we observed no difference between the results of the social-empty and social-object conditions. Moreover, comparing exploration dynamics of the empty enclosure in social-empty and the object-containing enclosure in social-object conditions, experimental mice displayed a non-significant decrease in the exploration of the object-containing enclosure across time compared to the empty one. Together, these results showed that an inanimate object did not affect social preference and/or may be assimilated as a neutral value across time. Since the experimental mouse explored the yellow rectangular box and moved it in the enclosure, some noise was emitted from hits of the box against the bars of the enclosure. It is therefore not excluded that this noise surprised the mouse and scared it after a couple of minutes, resulting in a decreasing interest for the inanimate object.

Finally, even though mice spent more time with an anesthetized mouse than exploring an object, they exhibited a significant decrease of exploration of the anesthetized conspecific whereas no change in object exploration was observed. Thus, the anesthetized mouse seems to become less attractive across time, even more than the inanimate object. A possible explanation could be that olfactory, tactile and visual characteristics of the anesthetized mouse provide curiosity and motivation to the experimental mouse. Only in a second time, the experimental mouse realizes that its conspecific is in an abnormal/weakness state rendering it unable to participate in the social interaction. As a

result, the loss of interest to interact with an anesthetized conspecific may be due to the frustration yielded by the impossibility to engage a previously expected bidirectional social interaction.

These experiments have some limitations, which will be discussed in the following paragraphs. The first limitation is the way of scoring the interaction time between the experimental mouse and the different stimuli, as it was performed manually and moreover, it was not possible, due to the nature of the experiment to do it in a blind way. Although manual scoring has certain advantages, like accuracy because sniffing time is specifically scored, it would be useful to automatically track these experiments. Another possible caveat could be that the induced animal anaesthesia was not complete in all of the mice. Even though the dose of 50mg/kg is assumed to induce a complete anesthetization, one fifth of injected mice presented tonic-clonic movements during the procedure, especially at the middle-end of the experiment. And although we can exclude that these mice were in an alert state, the anesthesia could be improved in future experiments to avoid these involuntary movements in all of the mice.

The concept of rewarding properties of social interaction is crucial for studying social motivation hypothesis in ASDs models. And although the differential time exploration between a social target and an empty enclosure provides information about social preference, the question whether social interactions are rewarding enough to promote an associative learning, in which environmental cues become progressively more attractive because they are assimilated to rewarding social interactions, remains open. Social conditioning place preference is often employed to test this hypothesis. However, nobody showed that when the contingencies were not kept between associations of stimuli and chambers no sCPP was found. For this reason, another goal of this study was to confirm the relevant impact of the conditioning sessions to produce a sCPP using a group that were tested under these conditions. As expected, these mice could not develop a preference for any chamber. The comparison between the group that underwent contingency break experiment and the paired conditioned-one showed a significant difference in the post-test session but not in the pre-test one. This emphasises the relevant role of the conditioning/pairing sessions to prefer one particular compartment because it was associated with a rewarding experience.

As social interaction can have aversive aspects, we performed a sCPA experiment using as social stimulus an aggressive CD1 mouse. In this case, a compartment was associated as a dangerous place for the paired conditioned group of mice. On the other hand, under the contingency break condition, the pairing of the chambers with the aggressive mice was not consistent. As expected, the results were the opposite compared to the ones obtained in sCPP experiment, which means that the exploration of the aggressor-paired chamber decreased after conditioning. However, the differential number of entries in the pairing compartment between pre-test and post-test sessions was discrepant between both experiments. Actually, while in sCPP there was no preference according to the number of entries, in sCPA this differential number of entries was significantly lower in the post-test session. In

order to assess whether the interaction with a CD1 mouse is more salient than with a social conspecific we compared the aversion score and the preference score respectively in the post-test session. These scores were identical, suggesting no difference between both opposite stimuli in the saliency to promote an associative learning. However, even though the total time conditioning period was similar in both experiments, the duration of conditioning sessions as well as their number differed between both experiments. Therefore, because the number of conditioning sessions is a more crucial parameter than their duration to produce a sCPP (35,42), it is difficult to compare both stimuli.

Several studies used protocols in which each conditioning session lasted 10 to 15 minutes. For the first time we showed that only 5-minute long conditioning sessions, but repeated several times throughout the day, were sufficient to produce a strong sCPP. Moreover, in the sCPA experiment, only one-minute long conditioning sessions produced a sCPA.

Our last goal was to determine whether the specific downregulation of SHANK3 in VTA would induce social impairments. Using the social-empty condition of the three chambers task described above, we showed that shShank3 mice did not exhibit any development of social preference and showed a time-dependant loss of interest for the social stimulus. Moreover, compared to control mice, experimental mice showed an increased grooming in the last session interval, suggesting a time-dependant increase of repetitive behaviour. It is therefore worth to note that the shShank3 mouse model shows social deficits as well as repetitive behaviours that are both ASDs core symptoms. Furthermore, we showed that the expression of these two symptoms was time-dependant, suggesting that the loss of interest for the social target is happening in parallel to an increase in the stereotyped repetitive behaviours. Further investigation and analyses of these two distinct behavioral outcomes should delineate their correlation, aiding us in the quest to better comprehend the symptom domains in ASDs.

Finally, since anxiety is often comorbid in ASD individuals, we wanted to assess whether VTA-*SHANK3* insufficiency would result in increased anxiety-like behavior. We use the Elevated Plus Maze paradigm to compare the same groups as described above. ShShank3 mice did not display any increased level of anxiety compared to scrShank3 mice. As a result, even though the VTA plays a role in mediating anxiety (46), downregulation of SHANK3 in this area does not promote an augmented avoidance of open arms. However, in order to confirm that SHANK3 downregulation in the VTA does not play a critical role in altering anxiety-like behavior, it would be important to perform other experiments measuring anxiety such as the open field and the light-dark test with this ASD mouse model.

Even though a variety of SHANK3 KO mice showed ASD related symptoms, little is known about the circuits-dependant function of this excitatory synapse post-synaptic protein. Here, we confirm that the specific downregulation of SHANK3 in VTA results in social deficits and increased

repetitive behaviours. Assessing the anxiety level of shShank3 mice, we better characterized this ASD mouse model. Together, these results confirm the crucial role of SHANK3 in the VTA to modulate social interaction and repetitive behaviours and thus suggest that the VTA-shShank3 mouse model is suitable for further studying ASD-related symptoms in order to better understand the underlying mechanisms and causes. Since shShank3 mice did not display a clear social preference, the next step would be to subject these mice to the sCPP task to confirm the non-rewarding properties of social interaction for this ASD mouse model. In addition, even though shShank3 did not show any increased anxiety in EPM, it would be interesting to address whether they would be more sensitive to social anxiety and defeat by performing the sCPA task. Moreover, other circuit-specific downregulation of SHANK3 should be performed in order to further elucidate its function at a circuit and systems level.

Moreover, DA neurons widely project in downstream corticolimbic areas. It would therefore be interesting to study their specific function in social behaviour and in the motivational aspects of social interaction. Since VTA to nucleus accumbens (NAc) projections are necessary and sufficient to promote social interactions (80), further investigations should be performed to determine the role of NAc in social motivation.

Recently, it was demonstrated that a positive allosteric modulator of mGluR1 ameliorated the social preference in shShank3 mice in long-lasting manner even if its administration was stopped at early adolescence (81). This emphasizes the great importance of early diagnostics (i.e. during synapse maturation) to propose new specific compounds in conjunction with the current behavioural therapies to optimize social integration of autistic individuals. However, since discrepancies between humans and rodents with *SHANK3* haploinsufficiency have been described, further translational studies have to be performed to compare the species-specific function of this post-synaptic protein.

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