Role of VTA dopamine neurons and neuroligin 3 in sociability traits related to nonfamiliar conspecific interaction

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Atypical habituation and aberrant exploration of novel stimuli have been related to the severity of autism spectrum disorders (ASDs), but the underlying neuronal circuits are unknown. Here we show that chemogenetic inhibition of dopamine (DA) neurons of the ventral tegmental area (VTA) attenuates exploration toward nonfamiliar conspecifics and interferes with the reinforcing properties of nonfamiliar conspecific interaction in mice. Exploration of nonfamiliar stimuli is associated with the insertion of GluA2-lacking AMPA receptors at excitatory synapses on VTA DA neurons. These synaptic adaptations persist upon repeated exposure to social stimuli and sustain conspecific interaction. Global or VTA DA neuron-specific loss of the ASD-associated synaptic adhesion molecule neuroligin 3 alters the behavioral response toward nonfamiliar conspecifics and the reinforcing properties of conspecific interaction. These behavioral deficits are accompanied by an aberrant expression of AMPA receptors and an occlusion of synaptic plasticity. Altogether, these findings link impaired exploration of nonfamiliar conspecifics to VTA DA neuron dysfunction in mice.
from infancy, we encounter an array of diverse stimuli from the environment. Repeated exposure to a stimulus can result in habituation whereas nonfamiliar stimuli usually increases exploratory behavior. Habituation and novelty recognition allow us focusing attention on what is unknown, promote exploratory behavior, facilitate learning, and are predictive of cognitive function later in life. Several neuropsychiatric disorders are characterized by deficits in habituation and novelty exploration. In autism spectrum disorder (ASD), young patients show prolonged attention to depictions of objects, but reduced attention to social stimuli. Moreover, ASD patients are hyporesponsive to novel visual stimuli and exhibit slowed habituation to faces. Such alterations are observed in a significant number of individuals with ASD, as they have been reported in clinical studies using diverse stimuli and read-outs. However, the circuits and neuronal mechanisms underlying this specific aspect of the ASD phenotype remain largely unknown.

Dopamine (DA) neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) may contribute to the habituation to familiar stimuli and to the exploration of nonfamiliar stimuli. DA neurons increase their activity in response to novel environments, to stimuli of positive or negative value, and to natural rewards. Interestingly, these neurons also respond to nonrewarding novel stimuli and their responses habituate when the stimulus becomes familiar. This has led to the proposal that novelty by itself may be rewarding. In rodents, nonfamiliar conspecífics or nonfamiliar objects increase Ca
transients in VTA DA neurons and this activity is necessary to promote social, but not object exploration. Glutamatergic synapses onto DA neurons undergo several forms of synaptic plasticity that may contribute to the modification of social interactions in response to experience. Specific synaptic adaptations have been described during development, after drug exposure, cue-reward learning, reciprocal social interactions, and after repeated burst stimulation of DA neurons. Furthermore, glutamatergic transmission is altered in several ASD animal models, and we have recently shown that deficits in the postnatal development of excitatory transmission onto VTA DA neurons lead to sociability deficits. Notably, several studies highlight decreased social reward processing in patients with ASD, and these alterations have been hypothesized to precipitate further developmental consequences in social cognition and communication. Whether specific forms of synaptic plasticity in the VTA are induced by exposure to nonfamiliar stimuli (novelty-induced synaptic plasticity), and whether aberrant plasticity associated with exploration of nonfamiliar conspecific in the VTA is related to the maladaptive responses in ASD mouse models is still largely unknown.

In this study, we parse the response to and the preference for nonfamiliar conspecifics as specific aspects of sociability controlled by DA neurons. We demonstrate that intact VTA DA neuron excitability is necessary to express a preference for nonfamiliar conspecifics but not for nonfamiliar objects. Additionally, we adopt a conditioned place preference protocol, based on interaction with familiar or nonfamiliar conspecific, to demonstrate that VTA DA neuron function underlies the reinforcing properties of social interaction. Mice lacking the ASD-associated synaptic adhesion molecule neuroligin 3 (Nlgn3) exhibit aberrant exploration of nonfamiliar conspecifics as well as deficit in habituation processing. These phenotypes are recapitulated by VTA DA neuron-specific down-regulation of Nlgn3. Finally, we discovered a form of novelty-induced synaptic plasticity at glutamatergic inputs onto VTA DA neurons that sustains conspecific interactions and is impaired in Nlgn3 KO and Nlgn3 VTA DA knockdown mice.

**Results**

**VTA DA neurons and exploration of nonfamiliar conspecifics.** Mice have been reported to interact with their conspecifics, to habituate upon repeated contact with the same subject, and to exhibit increased exploration when subsequently brought into contact with a nonfamiliar mouse. To examine whether VTA DA neurons regulate exploration of nonfamiliar conspecifics, we examined the behavior of mice in which the inhibitory DREADD (hM4Di) or mCherry were virally expressed in DA neurons of the VTA (VTA::DAhM4Di; AAV5-hSyn-DIO-hM4Di-mCherry or VTA::DAmCherry; AAV5-hSyn-DIO-mCherry injected into DATCre mice, Fig. 1a). Virus infusions led to mCherry expression in 50% of TH+ (tyrosine hydroxylase, an enzyme necessary for DA synthesis) VTA neurons and in few (2%) of TH+ cells in the neighboring substantia nigra pars compacta (SNc; Supplementary Fig. 1a), confirming preferential targeting of the VTA. Application of the hM4Di ligand clozapine-n-oxide (CNO) decreased the neuronal excitability of VTA::DAhM4Di neurons compared to VTA::DAmCherry ex vivo (Supplementary Fig. 1b) and decreases DA release in striatal regions in vivo.

We then assessed the time spent in social interaction upon repeated exposure to the same mouse (habituation) and the subsequent response to a nonfamiliar conspecific (Fig. 1b). To compare between social and nonsocial stimuli, we also examined the behavioral responses to familiar and nonfamiliar objects (Supplementary Fig. 1c–f). When repeatedly exposed to the same mouse (Fig. 1c) or object stimulus (Supplementary Fig. 1c; s1 and o1, respectively), VTA::DAhM4Di animals injected with vehicle showed unaltered stimulus exploration (s2, Fig. 1e, f). Interestingly, when exposed to a nonfamiliar object (o2), both VTA::DAhM4Di and VTA::DAmCherry animals treated with CNO exhibited an increased exploration (Supplementary Fig. 1e, f). Thus, reducing VTA DA neuron excitability specifically alters the exploration of a nonfamiliar conspecific, but not of a nonfamiliar object, suggesting a differential requirement of DA neuron activity for driving exploration of social and inanimate stimuli.

**VTA DA neurons and preference for nonfamiliar conspecifics.** To assess the role of VTA DA neuron excitability in mediating the exploration of a nonfamiliar conspecific over an inanimate object or a familiar conspecific stimulus, VTA::DAhM4Di and VTA::DAmCherry mice were subject to the three-chamber test under vehicle and CNO conditions. The test was performed twice: first, animals received either vehicle or CNO and, after 1 week of washout, the test was repeated and the pharmacological treatment was counterbalanced (Fig. 2a). To monitor potential off target effects of CNO, we also included VTA::DAmCherry mice treated with CNO as controls. During the task, test mice were given a choice between an object (o1) versus a nonfamiliar mouse (s1 or s3) and subsequently a choice between a familiar (second exposure to s1 or s3) versus a nonfamiliar conspecific (s2 or s4).

Previous studies define sociability in this assay as longer time spent in the chamber with the same-sex nonfamiliar mouse rather
VTA DA neurons and nonfamiliar conspecific reinforcement.

To investigate whether nonfamiliar conspecific interactions are reinforcing in mice, we performed a conditioned place preference (CPP) task (modified from 31, 32). Briefly, test mice are housed with familiar mice throughout the protocol. After the Pre-TEST, we performed 4 days of repeated conditioning where wild-type (WT) mice learn to associate one compartment of the apparatus with the presence of either a familiar conspecific (familiar, f) or a nonfamiliar object (o1) stimulus, while the other compartment is left empty (Fig. 3a,b). At day 5 (Post-TEST) the preference to explore the two compartments, in across groups, we calculated a “social novelty index,” as time spent avoiding the nonfamiliar stimulus minus time spent exploring the familiar target, in the first and last 5 min of the assay. We found that the social novelty index was reduced by CNO injections in VTA::DAH4Di mice compared to both CNO treated VTA::DAmCherry and vehicle treated VTA::DAH4Di (Fig. 2i). Altogether, these findings indicate that reducing the excitability of DA neurons decreases the exploration of novel social stimuli, when given a choice between nonfamiliar and familiar conspecics.
absence of any stimulus, was quantified and compared to Pre-
TEST. While no significant preference was developed for the
familiar conspecifics (Fig. 3c and Supplementary Fig. 3a), mice
exhibited preference for the compartment associated with the
nonfamiliar conspecifics (Fig. 3d and Supplementary Fig. 3b), and
an avoidance for the novel object stimulus associated chamber
(Fig. 3e and Supplementary Fig. 3c). Interestingly, across con-
ditioning sessions, we observed habituation to all the stimuli
(Fig. 3f–h). However, when the time of interaction with the sti-
mulus during the first and the last day of conditioning was

![Diagram showing 3-chamber test with objects and stimuli]
analyzed, we observed a longer interaction with the nonfamiliar conspecifics compared to the other stimuli at either time point (Fig. 3i). These data suggest that a nonfamiliar conspecific remains salient over days and promotes contextual associative learning.

To assess the role of VTA DA neuron excitability in mediating the reinforcing properties of nonfamiliar conspecific interactions, both control VTA::DAmCherry and VTA::DAhM4Di received injections of CNO before each conditioning session and were treated with vehicle before the Post-TEST (Fig. 3j). Control VTA::DAmCherry but not VTA::DAhM4Di mice developed a preference for the compartment associated with the nonfamiliar conspecifics (Fig. 3k and Supplementary Fig. 3d, e). These observations suggest that the excitability of DA neurons mediates both the interaction with nonfamiliar conspecifics as well as the acquisition of nonfamiliar conspecific-induced contextual associations.

Altered conspecific interactions in Nlgn3KO mice. Patients with ASD exhibit slowed habituation to faces and are less responsive to social reward32. Thus, we tested whether a deletion of Nlgn3 in mice, a category 2 (strong candidate) classified ASD-linked gene (http://gene.sfari.org)33–35 encoding a postsynaptic adhesion molecule36, might result in deficits in recognition of nonfamiliar conspecifics and in the reinforcing properties of conspecific interaction. Global Nlgn3KO mice37 exhibit reduced ultrasonic vocalization and social memory in male–female interactions as well as altered motor behaviors and olfaction38–41. We examined the interaction time upon repeated exposure to a familiar mouse (habituation) and the subsequent response to a nonfamiliar conspecific (Fig. 4a). Nlgn3KO mice exhibited overall lower interaction times, no significant habituation, and lacked the increased response to nonfamiliar conspecifics seen in Wild Type (WT) littermates (Fig. 4b, c and Supplementary Fig. 4a–d). However, Nlgn3KO mice showed habituation, increased exploration of nonfamiliar objects (Fig. 4d, e) and preference for nonfamiliar objects in a novel object recognition task (Fig. 4f–h). This indicates that both novelty preference and memory for objects are unaltered. In addition to impaired response to nonfamiliar conspecifics, Nlgn3KO mutants exhibit alterations in motor activity (Fig. 4i) and marble burying (Fig. 4j). In an olfactory discrimination test32, Nlgn3KO male mice showed normal response and habituation to a social odor (Supplementary Fig. 4e). However, the mutant mice had a significantly decreased response when subsequently presented to a second (novel) social odor (Supplementary Fig. 4e). To further examine conspecific interaction in Nlgn3KO mice, we tested the reinforcing properties of social interaction31,32. When mice are conditioned in a conditioned place preference paradigm with familiar mice, Nlgn3KO mice did not develop a preference for the social compartments, whereas WT mice did (Fig. 4k, l, and Supplementary Fig. 4f, g). These findings suggest that Nlgn3KO mice exhibit altered social interactions and defects in social reward behaviors.

Nlgn3 loss-of-function in VTA DA neurons alters sociability. The diverse alterations in social but also nonsocial behaviors in Nlgn3KO mice, indicate that multiple different systems might contribute to their phenotype. To test whether any alterations are due to Nlgn3 functions in VTA DA neurons we generated microRNA-based knock-down vectors for conditional suppression of Nlgn3 expression (Supplementary Fig. 5a, b). CRE-dependent AAV-based vectors were injected into the developing VTA of DAT-Cre mice at postnatal days 5–6 and mice were analyzed using a battery of behavioral tests (AAV2-DIO-miR35 in DAT-Cre mice: VTA::DANl3KD, Fig. 5a, b, and see Supplementary Fig. 5c for off-target areas affected and Supplementary Fig. 5d, e for further controls). Notably, VTA::DANl3KD mice exhibited a similar impairment in reinforcing properties of conspecific interaction as the global Nlgn3KO mice in the conditioned place preference paradigm (Fig. 5c, d, and Supplementary Fig. 5f, g) indicating that Nlgn3 downregulation in VTA DA neurons is sufficient to mimic this aspect of the global Nlgn3KO phenotype. Furthermore, when repeatedly exposed to the same and subsequently to a nonfamiliar conspecific, VTA::DANl3KD mice showed an overall reduction in social exploration and a blunted response to novel conspecific stimuli (Fig. 5e–g, and Supplementary Fig. 5h–j). At the same time, VTA::DANl3KD mice showed preference for novel objects in the novel object recognition task (Fig. 5k–l). Thus, there is a specific requirement for Nlgn3 in VTA DA neurons for appropriate exploration of nonfamiliar conspecifics and for the reinforcing properties of social interaction. By contrast, motor activity, marble burying, and social olfaction that are altered in global Nlgn3KO mice were not modified in the VTA::DANl3KD mutants (Fig. 5k, l, Supplementary Fig. 5l). Interestingly, we observed that knock-down of Nlgn3 in VTA DA neurons of adult mice produced a similar but less pronounced social interaction phenotype as in developing animals, with reduced habituation and reduced response to nonfamiliar conspecifics (Supplementary Fig. 6). Thus, Nlgn3 expression, in both developing and mature VTA DA circuits, is required for habituation and nonfamiliar conspecific exploration.

A synaptic signature of saliency detection in VTA DA neurons. Several experiences strengthen synaptic transmission at excitatory inputs onto DA neurons and drive the insertion of GluA2-lacking
AMPARs, which can be assessed by calculating a rectification index (RI)\(^1\). We tested whether nonfamiliar exploration induced specific forms of long-lasting synaptic plasticity at excitatory inputs onto DA neurons in the VTA (novelty-induced synaptic plasticity). In WT mice, the RI increased at synapses 24 h after the exploration of either a nonfamiliar mouse or a nonfamiliar object when compared to RI calculated from home caged mice (Fig. 6a). By contrast, the RI was unchanged after the exposure to a new context and AMPA/NMDA ratios were unchanged for any of the above conditions (Fig. 6b). When AMPAR EPSCs were recorded after repeated exposure (over 4 days) to object stimuli, the RI was normalized to control condition (Fig. 6c). A subsequent exposure

![Diagram](image-url)
to a new object (o2) increased the RI (Supplementary Fig. 7a). By contrast, GluA2-lacking AMPARs were detected in mice repeatedly exposed to a nonfamiliar conspecific stimulus (s1) over a 4-day period and were still present at these synapses after 10 days of repeated exposure (Fig. 6c). Remarkably, the AMPA/NMDA ratio was significantly elevated after 4 days of social (s1) repeated exposure relative to baseline but was normalized after 10 days of repeated exposure (Fig. 6d), while the paired-pulse ratio (PPR) remained unchanged throughout (Supplementary Fig. 7b). Taken together, these data indicate that repeated exposure to a nonfamiliar conspecific stimulus, but not an object stimulus, transiently increases synaptic strength (AMPA/NMDA ratio) and produces a stable insertion of GluA2-lacking AMPARs at VTA DA neuron excitatory inputs.

To understand the functional role of noncanonical AMPARs inserted during nonfamiliar conspecific exposure, we infused the GluA2-lacking AMPAR blocker NASPM into the VTA starting from the second day of interaction with either social or object stimuli (Fig. 7a, b). NASPM infused mice reduced the interaction with a conspecific stimulus upon repeated exposure (Fig. 7c); by contrast, the infusions did not alter long-term habituation to an object (Fig. 7d), interaction in the home cage between two familiar mice or distance moved in an open field (Supplementary Fig. 7c–e). To further understand the impact of GluA2-lacking AMPARs at VTA DA neuron inputs on conspecific repeated exposure, we promoted the insertion of GluA2-lacking AMPARs via blue-light illumination of ChR2 or eYFP expressing VTA DA neurons18 of DAT-Cre mice (VTA::DAChR2: AAV5-Ef1-DIO-ChR2(H134R)-eYFP, VTA::DAeYFP: AAV5-Ef1-DIO-eYFP). DA neuron stimulation consisted in 15-minute long ChR2-mediated bursts of action potentials18 delivered the day before each conspecific exposure (Fig. 7e, f). This noncontingent burst activation increased RI in photocurrent positive neurons (I Chlor; Fig. 7g) and blocked habituation to social stimuli (Fig. 7h). Altogether, these data indicate that GluA2-lacking AMPARs might represent a synaptic signature of conspecific saliency and, once inserted, their activity counteracts habituation.

**Discussion**

In this study, we establish that intact VTA DA neuron excitability is necessary for (1) the exploration of nonfamiliar social stimuli, (2) the preference for nonfamiliar versus familiar conspecifics, and (3) the acquisition of nonfamiliar conspecific-induced contextual associations. Novel stimuli, independent of their nature, leave a plasticity trace at glutamatergic synapses in the VTA, which persists upon repeated exposure to social stimuli and supports sustained conspecific interactions. We use a deletion of the ASD-associated gene Nlgn3 and demonstrate that global Nlgn3 knock-out results in an impaired habituation and an aberrant exploration of nonfamiliar conspecifics. Furthermore, selective inactivation of Nlgn3 in VTA DA neurons disrupts novelty-induced plasticity at glutamatergic synapses in the VTA, alters exploration of nonfamiliar conspecific, and the reinforcing properties of conspecific interactions while having no detectable effect on motor behaviors or olfaction.

Global loss of Nlgn3 is also accompanied by a broad spectrum of additional phenotypes, including changes in olfaction and in motor-related behaviors38–41. Thus, the origin of social behavior alterations in these mice was unclear. Previous studies explored phenotypes in mice carrying a point mutation in Nlgn3 that reduces (but does not abolish) Nlgn3 expression and has been observed in 2 patients from one family43. For this model, it was concluded that behavioral phenotypes are significantly dependent on the genetic context with significant phenotypes reported for some genetic backgrounds but not others44–46. Our study demonstrates that VTA DA neuron specific Nlgn3 loss of function is sufficient to recapitulate sociability deficits reported in global KO mice.

Although several studies have provided instrumental information about the neuronal circuits, within the reward system, that control social behavior in rodents13,47–49, the synaptic

**Fig. 3** VTA DA neuron excitability mediates the reinforcing properties of nonfamiliar conspecific. a Experimental protocol for conditioned place preference with different stimuli. b Representative occupancy plots. c Scatter plot of preference score measured for familiar mouse pairing during CPP. Paired t test (*t*<sub>9</sub> = 2.0886; mean and s.e.m for Pre-TEST: 0.498 ± 0.0298; mean and s.e.m for Post-TEST: 0.506 ± 0.0562). d Scatter plot of preference score for nonfamiliar conspecific pairing during CPP. Paired t test (*t*<sub>9</sub> = 4.578; mean and s.e.m for Pre-TEST: 0.497 ± 0.0144; mean and s.e.m for Post-TEST: 0.596 ± 0.0285). e Scatter plot of preference score for novel object pairing during CPP. Paired t test (*t*<sub>9</sub> = 2.263; mean and s.e.m for Pre-TEST: 0.510 ± 0.0430; mean and s.e.m for Post-TEST: 0.403 ± 0.0455). f Time course of interaction during conditioning blocks with a familiar mouse (f). Friedman test (P = 0.0150; x<sup>2</sup><sub>5</sub> = 23.51). g Time course of interaction during conditioning blocks with a nonfamiliar conspecific (g). Friedman test (P < 0.0001); x<sup>2</sup><sub>5</sub> = 52.71). h Time course of interaction during conditioning blocks with a novel object (h). Friedman test (P = 0.0008; x<sup>2</sup><sub>12</sub> = 31.88). i Cumulative interaction during conditioning sessions at day 1 and day 4, respe-Wallis test (K = 46.09, P < 0.0001) followed by Dunn’s test for planned comparisons. j Left: experimental protocol for VTA::DA<sup>M4D4</sup> and VTA::DA<sup>mCherry</sup> treated with CNO during CPP with nonfamiliar conspecific pairings. Right: representative occupancy plots for CNO treated VTA::DA<sup>mCherry</sup> and VTA::DA<sup>M4D4</sup>. k Scatter plot of preference score for VTA::DA<sup>mCherry</sup> treated with CNO during conditioning sessions with a nonfamiliar conspecific (mean and s.e.m for Pre-TEST: 0.4808 ± 0.0267; mean and s.e.m for Post-TEST: 0.5836 ± 0.0275), and scatter plot of preference score for VTA::DA<sup>M4D4</sup> treated with CNO during conditioning sessions with a nonfamiliar conspecific (mean and s.e.m for Pre-TEST: 0.4903 ± 0.0162; mean and s.e.m for Post-TEST: 0.5091 ± 0.0428). RM two-way ANOVA (time main effect: F<sub>1, 24</sub> = 7.7048, P = 0.0105; virus main effect: F<sub>1,24</sub> = 0.8678, P = 0.3609; time × virus interaction: F<sub>1,24</sub> = 3.2861, P = 0.0824) followed by Bonferroni post hoc test for planned comparisons. N indicates number of mice. Error bars represent s.e.m.
adaptations occurring at VTA DA neurons during interactions with nonfamiliar conspecifics remained largely unknown. Here, we show that while reduced excitability or conditional suppression of Nlgn3 in VTA DA neurons affect the exploration of nonfamiliar conspecifics, they both fail to modify responses to novel objects, presumably because of the higher intrinsic salience of social stimuli. Consistent with this hypothesis, we observe that while both nonfamiliar object and conspecific exploration trigger the insertion of GluA2-lacking AMPARs, only the repeated exposure to the same nonfamiliar mouse results in the maintenance of GluA2-lacking AMPARs at glutamatergic synapses of VTA DA neurons. Therefore, we hypothesize that while the insertion of non-canonical AMPARs reflects the novelty associated to the stimulus, their persistence signals the higher salience of the conspecific over the object stimulus. The insertion and the expression of noncanonical AMPARs has been previously associated with nonsocial and highly salient experiences, such as cocaine exposure\cite{ref17}. However, a causal relationship between behavioral responses to salient stimuli and GluA2-lacking AMPAR expression at VTA DA neurons has not been
Fig. 5 Nlgn3 in VTA DA neurons is required for social exploration and the reinforcing properties of conspecific interaction. a Left: representative image of coronal slice of VTA and SNc from an AAV2 DIO-miRNA3-GFP infected DAT-Cre mouse. Right: higher magnification of VTA. Scale bar: 1 mm and 100 μm. b Experimental schematic of behavioral test order in VTA-injected mice. c Experimental schematic of the social-CPP test. d Scatter plot of preference score measured during the Pre- and Post-TEST for VTA::GFP (mean and s.e.m for pre-TEST = 0.4642 ± 0.0247. Mean and s.e.m post-TEST = 0.5526 ± 0.0200), and VTA::DA NL3KD mice (mean and s.e.m for Pre-TEST: 0.4434 ± 0.0218; mean and s.e.m for Post-TEST: 0.4548 ± 0.0214). RM two-way ANOVA (time main effect: F(4, 80) = 2.908, P = 0.0237; virus main effect: F(1, 20) = 9.164, P = 0.0024) followed by Bonferroni’s post hoc test for planned comparisons. e Experimental schematic of the habituation/nonfamiliar exploration task. f Mean social interaction plotted for VTA::GFP and VTA::DA NL3KD mice. RM two-way ANOVA (time main effect: F(4, 80) = 8.058, P < 0.0001; virus main effect: F(1, 20) = 9.164, P = 0.0007; time × virus interaction: F(4, 80) = 3.179, P = 0.0178) followed by Bonferroni’s post hoc test. g Social novelty index for VTA::GFP and VTA::DA NL3KD mice. Unpaired t test (t(120) = 2.908). h Experimental schematic of novel object recognition test. i Time spent investigating a novel and a familiar object. Paired t test (VTA::GFP: t(13) = 3.763. Mean and s.e.m familiar = 6.199 ± 0.805, mean and s.e.m novel = 14.03 ± 2.188. VTA::DA NL3KD: t(7) = 6.518. Mean familiar = 8.226, s.e.m ± 1.069, mean novel = 16.2, s.e.m ± 1.582). j Discrimination ratio for object discrimination plotted for VTA::GFP and VTA::DA NL3KD mice during a 7 min open field test. Mann–Whitney U = 2.908. k Number of marbles buried plotted for VTA::GFP and VTA::DA NL3KD. Mann–Whitney U = 49.5. N numbers indicate mice. All error bars are s.e.m.
reported. Here, we show that the insertion of noncanonical AMPARs at VTA DA neurons contributes to behavioral responses to social stimuli suggesting that these receptors could also represent a functionally-relevant synaptic signature responsible for the behavioral responses associated with other salient stimuli.

Changes in AMPA/NMDA ratio occur in response to both rewarding and aversive processes\(^\text{30}\), and synaptic strengthening is transiently expressed and necessary for associative learning\(^\text{31}\). Consistent with previous findings\(^\text{16}\), we report an increased AMPA/NMDA ratio at VTA DA neuron excitatory inputs in response to social interaction, which is transiently expressed upon repeated exposure to nonfamiliar conspecifics, but not object stimuli. However, although the increased synaptic strength might represent an additional signature related to the saliency of social interaction, its role in habituation processing and, possibly contextual learning, warrants further investigation.

In recent years, accumulating evidences indicate that synaptic adaptations associated to reward and aversion occur at projection-specific subclasses of VTA DA neurons\(^\text{32,33}\). An anatomic-functional segregation of reward circuitry is also emerging in respect of social behavior: while VTA DA neurons projecting to the nucleus accumbens (NAc), but not prefrontal cortex (PFC), promote conspecific interaction\(^\text{13}\), DA neuron projections to the interpenduncular nucleus control familiarity signaling\(^\text{47}\). Thus, the specific synaptic signatures observed in response to nonfamiliar conspecific exposure might also occur in dedicated VTA circuits. At the same time, given the intrinsic diversity of sensory and emotional information provided by social vs. inanimate stimuli, it is conceivable that synaptic plasticity occurs at specific inputs to defined subclasses of VTA DA neurons. Additional investigations of synaptic properties of defined inputs to projection-specific DA neuron subclasses is needed to further understand the circuits and the synaptic mechanisms

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**Fig. 6** Novelty-induced synaptic plasticity. **a** Top: experimental paradigm. Bottom: scatter plot of rectification index and AMPA-EPSCs example traces (–60, 0, and 40 mV) recorded from VTA DA neurons at baseline (B, homecage), or 24 h after 15 min of novel context (Nc), nonfamiliar conspecific (s1) or novel object (o1) exposure. One-way ANOVA (F\(_{(3, 42)} \text{= 5.149, } P = 0.0030\) follow by Bonferroni post hoc test for planned comparisons. **b** Top: experimental paradigm. Bottom: scatter plot and example traces of AMPA/NMDA ratio recorded from VTA DA neurons at baseline (B, homecage), or 24 h after 15 min of novel context (Nc), nonfamiliar conspecific (s1) or novel object (o1) exposure. One-way ANOVA (F\(_{(3, 42)} \text{= 0.0287, } P = 0.9933\)). **c** Top: experimental paradigm. Bottom: scatter plot and example traces of rectification index recorded from VTA DA neurons at baseline (B), 24 h after four repeated exposures to either a novel mouse (s1) or a novel object (o1) and ten repeated exposures to a nonfamiliar conspecific (s1, bold purple). One-way ANOVA (F\(_{(3, 46)} \text{= 4.4939, } P = 0.0076\) followed by Bonferroni post hoc test for planned multiple comparisons. **d** Top: experimental paradigm. Bottom: scatter plot and example traces of AMPA/NMDA ratio recorded from VTA DA neurons at baseline (B), 24 h after four repeated exposures to either a nonfamiliar conspecific (s1) or a novel object (o1) and ten repeated exposures to a nonfamiliar conspecific (s1, bold purple). One-way ANOVA (F\(_{(3, 46)} \text{= 4.4939, } P = 0.0076\) followed by Bonferroni post hoc test for planned multiple comparisons. n,N indicates number of cells and mice respectively. Scale bars: 20 msec, 20 pA. Error bars report s.e.m.
underlying both novelty and saliency processing associated with conspecific and inanimate stimuli.

Altered social interactions and communication are defining aspects of the autism phenotype. However, such alterations may arise from a plethora of neuronal processing defects, ranging from alterations in perception, sensory processing, multisensory integration, or positive and negative valence assigned to conspecific stimuli. In this work, we specifically explore neuronal circuitry relevant for the exploration to and the preference for nonfamiliar conspecifics. We chose this domain, as studies in children with ASD demonstrated altered habituation and responses to novel stimuli. Notably, in toddlers, a slowed habituation to faces but normal habituation to repeatedly viewed objects has been reported to coincide with more severe ASD symptoms. Several rodent models of ASD exhibit altered social memory or discrimination. However, brain areas and circuit elements contributing to these changes in social communication remain poorly understood. Here, we tested the hypothesis that GluA2-lacking AMPAR function controls habituation to nonfamiliar conspecics.

Fig. 7 GluA2-lacking AMPAR function controls habituation to nonfamiliar conspecics. a Schema of the experimental paradigm. b Representative image of cannula placement for NASPM or vehicle infusion (green: TH; blue: DAPI; white arrow indicates cannula tip). c Time course of time interaction with a nonfamiliar conspecific (s1) for vehicle or NASPM infused mice at day 2, day 3, and day 4. RM two-way ANOVA (time main effect: F(3, 24) = 17.57, P < 0.0001; drug main effect: F(1, 8) = 16.48, P = 0.0036; time × drug interaction: F(3, 24) = 3.141, P = 0.0439). d Time course of time interaction with a novel object (o1) over 4 days for Vehicle and NASPM groups. RM two-way ANOVA (time main effect: F(3, 33) = 24.71, P < 0.0001; drug main effect: F(1, 11) = 0.00005, P = 0.9942; time × drug interaction: F(3, 33) = 1.109, P = 0.3595). e Experimental paradigm for noncontingent optogenetic stimulation. f Representative image of optic fiber placement for DIO-ChR2 expressing mice (red: TH, green: AAV-DIO-ChR2-eYFP, blue: DAPI; white arrow indicates fiber optic tip). g Left: example traces of a photocurrent negative (I_{ChR2}^-) and a photocurrent positive (I_{ChR2}^+) VTA DA neuron. Scale bars: 20 msec, 1 nA. Middle: scatter plot of RI recorded from photocurrent negative (I_{ChR2}^-) and photocurrent positive (I_{ChR2}^+) VTA DA neurons and AMPAR-EPSCs example traces (−60, 0, and 40 mV) recorded from VTA DA neurons. Mann–Whitney test (U = 6). Scale bars: 20 msec, 20 pA. h Time course over 4 days of time interaction with a nonfamiliar conspecific (s1) for VTA::DAChR2 and VTA::DAeYFP mice with noncontingent optical stimulation. RM two-way ANOVA (time main effect: F(3, 18) = 2.9966, P = 0.0386; virus main effect: F(1, 18) = 7.9034, P = 0.0116; time × virus interaction: F(3, 18) = 1.9532, P = 0.1320). N indicates number of mice. Error bars represent s.e.m.
habituation and social novelty responses in mice and humans are largely known. Our rodent work not only highlights a contribution of VTA DA neurons to this process but also takes steps toward identification of the synaptic basis of social novelty responses and habituation. Considering the complexity of ASD behavioral dysfunctions, we propose that fractionating the autism phenotype according to specific behavioral domains based on neuronal circuit elements will provide a productive stratification criterion for patient populations. Thus, we speculate that in a subpopulation of individuals with ASD alterations in VTA DA function might contribute to the social interaction phenotype whereas in other subgroups of patients alterations in social interaction may arise for different reasons. A prediction from this hypothesis is that stratification of patient populations based on an assessment of novelty responses, habituation, and social reward may help to identify subgroups of patients that would particularly benefit from interventions targeting function and plasticity of the VTA-DA circuit elements.

Methods

Animals. The study was conducted with WT and transgenic mice in C57BL/6J background. WT mice were obtained from Charles River. For DA neuron-specific manipulations DAT-iresCre (SlokAgmt1.1x(cre)Bknny3) and DAT-Cre BAC transgenic mice were employed. Nlgn3KO mice were previously described37. Male and female mice were housed in groups (weaning at P21) – dehedated, and placed with a 10° angle, at following stereotaxic coordinates: ML ± 0.9 mm, AP – 3.2 mm, DV –60 mV. Injections of rAAV5-Ef1α-DIO-hChR2(H134R)-eYFP and rAAV5-Ef1α-DIO-hSyn-DIO-mCherry were done at P5–P6 for developmental knockdown and at 4–7 weeks for adult knockdown. For additional information on viral vectors, see Supplementary Methods. Mice were anesthetized and placed in a stereotactic frame (Angle One; Leica, Germany). The virus was injected via a glass micropipette (Drummond Scientific Company, Broomall, PA) into the VTA at the rate of 100 nl/min for a total volume of 200 nL in each side. The virus was incubated for 3–4 weeks prior to perform the behavioral tasks or electrophysiological recordings.

Injections of purified AAV2-DIO-miRNlg3-GFP, AAV2-Synaptophysin-GFP and AAV2-DIO-miR-GFP were done at P5–P6 for developmental knockdown and at 4–7 weeks for adult knockdown. For additional information on viral vectors and validation of construct for Neuroglin 3 downregulation, see Supplementary Methods. Mice were anesthetized with a mixture of oxygen and isoflurane anesthesia (Baxter AG, Vienna, Austria) as previously described. The animals were placed in a stereotactic frame (Kopf Instrument) and a single craniotomy was made over the VTA at following stereotaxic coordinates: ML ± 0.5 mm, AP –4.4 mm, DV –4.2 mm from Bregma. The virus was injected via a glass micropipette (Drummond Scientific Company, Broomall, PA) into the VTA at the rate of 100 nl/min for a total volume of 200 nL in each side. The virus was incubated for 3–4 weeks prior to perform the behavioral tasks or immunostaining. Mice were excluded from the study if the body weight was less than 75% of the mean body weight at the start of behavior trials.

Injections of rAAV5-Ef1α-DIO-hChR2(H134R)-eYFP and rAAV5-Ef1α-DIO-eYFP were performed in DAT-Cre mice at 4–5 weeks. For additional information on viral vectors, see Supplementary Methods. Mice were anesthetized and placed in a stereotactic frame (Angle One; Leica, Germany) as previously described. The skin was shaved, locally anesthetized with 40–50 µL lidocaine 0.5% and disinfected. Bilateral craniotomy (1 mm in diameter) was then performed over the VTA at following stereotaxic coordinates: ML ± 0.5 mm, AP –3.2 mm, DV –4.20 ± 0.05 mm from Bregma. The virus was injected via a glass micropipette (Drummond Scientific Company, Broomall, PA) into the VTA at the rate of 100 nl/min for a total volume of 200 nL in each side. The virus was incubated for 3–4 weeks prior to perform the behavioral tasks or immunostaining. Mice were excluded from the study if the body weight was less than 75% of the mean body weight at the start of behavior trials.

Surgery. Injections of rAAV5-hSyn-DIO-hM4D(Gi)-mCherry and rAAV5-hSyn-DIO-mCherry were performed in DAT-Cre mice at 4–7 weeks. For additional information on chemogenetic viral vectors, see Supplementary Methods. Mice were anesthetized with a mixture of oxygen (1 L/min) and isoflurane 3% (Baxter AG, Vienna, Austria) and placed in a stereotactic frame (Angle One; Leica, Germany). The skin was shaved, locally anesthetized with 40–50 µL lidocaine 0.5% and disinfected. Bilateral craniotomy (1 mm in diameter) was then performed over the VTA at following stereotaxic coordinates: ML ± 0.5 mm, AP –3.2 mm, DV –3.95 ± 0.05 mm from Bregma. The virus was implanted with a 10° angle, placed on the skull with dental acrylic. Implantations of stainless steel 26-gauge cannula (PlasticsOne, Virginia, USA) were performed on WT mice at 8–10 weeks. Mice were anesthetized and placed in a stereotactic frame as previously described. Unilateral craniotomy (1 mm in diameter) was then performed over the VTA at following stereotaxic coordinates: ML ± 0.9 mm, AP –3.2 mm, DV –3.95 ± 0.05 mm from Bregma. The cannula was implanted with a 10° angle, placed above the VTA and fixed on the skull with dental acrylic.

Fig. 8 Aberrant increase of GluA2-lacking AMPARs in Nlgn3-deficient VTA DA neurons. a Top: experimental paradigm. Bottom: scatter plot of rectification index and example traces of AMPAR-EPSCs (−60, 0, and 40 mV) measured from adolescent WT, Nlgn3KO and VTA::DA NL3KD. One-way ANOVA (F(2,23) = 8.363, P = 0.0019) followed by Bonferroni post hoc test. b Top: experimental paradigm. Bottom: scatter plot of rectification index and example traces of AMPAR-EPSCs (−60, 0, and 40 mV) measured from VTA::DANL3KD mice at baseline (B) or 24 h after 15 min exposure to a nonfamiliar conspecific (s1). Unpaired t test (t(16) = 0.7536). n, N indicates number of cells and mice respectively. Scale bars: 20 msec, 20 pA. Error bars represent s.e.m.

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dental acrylic. Between experiments, the cannula was removed by a removable cap. All animals underwent behavioral experiments 1-2 weeks after surgery.

**Three-chamber test.** The three-chambered social preference test was performed in a rectangular Plexiglas arena (60 × 40 × 22 cm) (Ugo Basile, Varese, Italy) divided into three chambers (each 20 × 40 × 22 cm) that communicate by removable doors situated on the walls of the center chamber. Three to eight weeks after virus infusions, VTA::DAhM4Di and VTA::DACherry mice were randomly assigned to two batches that received intraperitoneal injections of either saline (vehicle) or Chloroquine (40 mg/kg; Sigma) 30 min before the experiment (see Supplementary Methods). All injections were done 30 min before starting the experiment. One to two weeks after, the experimental subjects treated first with CNO received vehicle and vice versa, thus performing the task in both conditions. The habituation phase consisted in 10 min of free exploration of the empty arena, and the mouse was then kept in the center chamber by closing the removable doors. Two enclosures were placed in the centers of the side chambers. One enclosure was left empty (inanimate object, o1) and the other one contained a nonfamiliar social stimulus (novel juvenile mice C57BL/6, 3–4 weeks old, s1/s3 in vehicle or CNO condition). The doors were then removed and the experimental mice were allowed to explore the arena and the two enclosures during the test. The stimulus interaction was scored when the animal was oriented toward the object stimulus. The time interaction was used to calculate the Novelty Index as: InteractionDay 5 − InteractionDay 1, both for social and object habituation/nonfamiliar exploration task.

The experimental cage was cleaned with 5% ethanol solution and the bedding was changed after each session. For the experiments with pharmacological agents, mice were cannulated to allow the infusion of either saline or 1-Naphthylacetyl spermine trihydrochloride (NASPM), directly in the VTA. The habituation task was performed as previously described. NASPM or saline were infused using a Minipump injector (pump Elite 11, Harvard apparatus, US) with 500 nl of saline (2 ml of active injection at 250 nl min−1 rate, and 1 min at rest), 10 min before each trial. At day 1, mice received saline (vehicle) and at the second day of the habituation phase, mice received NASPM dissolved in 500 nl of saline or 500 nl of saline only (at 250 nl min−1) before each trial. This dose has been previously used to obtain GluA2-lacking AMPARs block in vivo[5]. After at least 1 week, the animals were re-tested to habituation/nonfamiliar exploration task and the pharmacological treatment was changed. The scoring of the social or object interaction was made as previously described. The experimental cage was cleaned with 5% ethanol solution and the bedding was changed after every session. To assess the cannula placement, experimental subjects were infused using Chicago Sky Blue 6B (1 mg ml−1), sacrificed 1–2 h later and transcardially perfused as previously described. For histology, the brains were removed and stained with cresyl violet, and sections were mounted on glass slides, dehydrated in ethanol, stained with cresyl violet, dehydrated again, and mounted with Entellan. Any visible deviation of the cannula trajectory within the apparatus and to manually score the time spent in nonaggressive interaction with the stimulus.

At day 0, experimental mice (male C57BL/6; group-housed; 8–16 weeks) freely explored the CPP apparatus for 15 min to determine Pre-Test preference for one of the chambers. The scoring of the social interaction during the habituation task was made as previously described. The scoring of the social interaction was made as previously described. The experimental cage was cleaned with 5% ethanol solution and the bedding was changed after every session. To assess the cannula placement, experimental subjects were infused using Chicago Sky Blue 6B (1 mg ml−1), sacrificed 1–2 h later and transcardially perfused as previously described. For histology, the brains were removed and stained with cresyl violet, and sections were mounted on glass slides, dehydrated in ethanol, stained with cresyl violet, dehydrated again, and mounted with Entellan. Any visible deviation of the cannula trajectory within the apparatus and to manually score the time spent in nonaggressive interaction with the stimulus.

**Long-term habituation/nonfamiliar exploration task.** An experimental cage similar to the animal’s home cage was used for this task. The bed was replaced after each trial and water and food were available. During the habitation phase (4 days, day 1–4), all VTA::DAhM4Di experimental mice received an intraperitoneal injection of saline 30 min before the task. The experimental VTA::DACherry mouse was placed in a nonfamiliar chamber (novel juvenile mouse, C57BL/6, 3–4 weeks old, s1). The animals were let free to explore the cage and to interact with each other for 15 min. At the end of the trial, the experimental and stimulus mice were returned to their home cage. For four consecutive days the experimental mouse was exposed to the same conspecific (s2/s4 in vehicle or CNO condition) and habituated to the environment during three sessions (30 min each) consisting of 30 min-long sessions (1 per day). Each session was subdivided in six blocks of 5 min during which the animals alternated between US+ and US− chamber, in presence of either familiar mouse, f1, nonfamiliar mouse, s1 or object novel, o1 or absence (empty) of the stimulus, respectively. Experimental mice were moved from the corridor during the alternations and returned to their home cage with their cage-mates at the end of the conditioning session. Groups were counterbalanced for US+/US− sequences and for dot or stripe wall pattern. VTA::DAhM4Di and VTA::DACherry mice received an intraperitoneal injection of NASPM (5 mg kg−1) 30–90 min prior each conditioning session. At day 5, during the Post-Test, experimental mice freely explored the CPP apparatus, without any stimulus for 15 min and the preference score was measured. The CPP apparatus was cleaned with 1% acetic acid, rinsed with distilled water and dried between each experimental subject.

**Social conditioned place preference.** Mice were tested at P30–P45 and were group-housed before the test. The test apparatus was a custom-built cage measuring 46 × 24 × 22 cm divided into three chambers. The two outer chambers (23 × 18 × 18 cm) were divided into horizontal and vertical striped pattern consisting or black rubber mats with different patterns (stripes vs. squares). The outer chambers were joined together by smaller chamber (23 × 10 cm) with white walls and floor with a 7 × 7 cm opening at the base to the outer chambers that can be closed. The cage was cleaned with 70% ethanol between each trial. During the pretrial, mice were left to freely explore the cage for 30 min. After the pretrial, all mice were single housed for the remainder of the test and one chamber was assigned the social chamber and one the isolation chamber. All mice received one social and one isolation condition session (30 min each) per day for 4 days, with a two-day rest between the second and third conditioning day. Mice were socially cohoused with their cage mates for 22 h and then habituated to the isolation chamber for 30 min. After the fourth conditioning day, mice were tested in a 30 min postconditioning trial. The time spent freely exploring the object was recorded as the social CPP score. The social CPP score was calculated as the duration of time spent in the social chamber (US+) − duration of time spent in the isolation chamber (US−). The preference score was calculated as time spent in stimulus chamber divided by the sum of time spent in stimulus chamber (US+) and the time spent in the empty chamber. No animals were excluded from the analysis based on preference score and US− pairwise comparisons were randomly assigned to dot or stripe chamber. At day 0, nonfamiliar conspecific (male C57BL/6; single-housed; 3–4 weeks) or familiar stimuli mouse (male C57BL/6; co-housed with experimental mice during the conditioning) were habituated to the US+ chamber for 15–30 min. The novel object stimulus was the same used in the object habituation/nonfamiliar exploration task. From day 1–4, experimental mice and controls received a conditioning schedule consisting of 30 min-long sessions (1 per day). Each session was subdivided in two blocks of 60 min during which the animals alternated between US+ and US− chamber, in presence of either familiar mouse, f1, nonfamiliar mouse, s1 or novel object, o1 or absence (empty) of the stimulus, respectively. Experimental mice were guided in the corridor during the alternations and returned to their home cage with their cage-mates at the end of the conditioning session. Groups were counterbalanced for US+/US− sequences and for dot or stripe wall pattern. VTA::DAhM4Di and VTA::DACherry mice received an intraperitoneal injection of NASPM (5 mg kg−1) 30–90 min prior each conditioning session. At day 5, during the Post-Test, experimental mice freely explored the CPP apparatus, without any stimulus for 15 min and the preference score was measured. The CPP apparatus was cleaned with 1% acetic acid, rinsed with distilled water and dried between each experimental subject.
Data availability. The data supporting this study are available upon request to the corresponding author.

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