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ORIGINAL INVESTIGATION

# Electrophysiological properties of dopamine neurons in the ventral tegmental area of Sardinian alcohol-preferring rats

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**Abstract**
*Rationale* Sardinian alcohol-preferring (sP) or -nonpreferring (sNP) rats are one of the few pairs of lines of rats selectively bred for their voluntary alcohol preference or aversion, respectively. Ventral tegmental area (VTA) dopamine (DA) neurons have long been implicated in many drug-related behaviors, including alcohol self-administration. However, the electrophysiological properties of these cells in sP and sNP rats remain unknown.
*Objectives* This study was designed to examine the properties of posterior VTA DA neurons and to unveil functional differences between sP and sNP rats.
*Materials and methods* The electrophysiological properties of DA cells were examined performing either single-cell extracellular recordings in anesthetized rats or whole-cell patch-clamp recordings in slices.
*Results* Extracellular single-unit recordings revealed an increased spontaneous activity in sP rats. However, a corresponding difference was not found in vitro. Moreover, DA cells of sP and sNP rats showed similar intrinsic properties, suggesting changes at synaptic level. Therefore, inhibitory- and excitatory-mediated currents were studied. A decreased probability of GABA release was found in sP rats. Additionally, sP rats showed a reduced depolarization-induced suppression of inhibition, which is an endocannabinoid-mediated form of short-term plasticity. Additionally, the effect of cannabinoid-type 1 (CB1) receptor agonist WIN55,212-2 on $GABA_A$ IPSCs was smaller in sP rats, suggesting either a reduced number or functionality of CB1 receptors in the VTA.
*Conclusions* Our findings suggest that both decreased GABA release and endocannabinoid transmission in the VTA play a role in the increased impulse activity of DA cells and, ultimately, in alcohol preference displayed by sP rats.

**Keywords** Alcohol · Dopamine neurons · Electrophysiology · Endocannabinoids · Sardinian alcohol-preferring (sP) rats · Ventral tegmental area · Cannabinoids

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## Introduction

The dopamine (DA) neurons originating in the ventral tegmental area (VTA) and projecting to both subcortical (e.g., nucleus accumbens, amygdala) as well as cortical (e.g., prefrontal cortex) structures form the mesocorticolimbic pathway (Fuxe et al. 1974; Oades and Halliday 1987). This mesocorticolimbic system has long been implicated in processing reward and controlling motivated behavior (Wise 2004). Indeed, both natural and pharmacological

stimuli (e.g., food, sex, drugs of abuse) have been shown to activate this system, which is, therefore, considered as the brain reward circuit (Esch and Stefano 2004). From an evolutionary point of view, these neurons mediating the rewarding effects of natural stimuli guarantee species preservation. Nonetheless, VTA DA cells also mediate the responses to conditioned stimuli and drugs of abuse (Marinelli et al. 2006; Melis et al. 2005; Pan et al. 2006; Wilson and Bowman 2006). In fact, changes in the spontaneous activity of VTA DA neurons, correlated with extracellular DA levels in the nucleus accumbens, are involved in the onset of drug addiction, as well as in the susceptibility of relapse (Marinelli et al. 2003). Particularly, addictive drugs, irrespective of their chemical nature, produce either an increase or a decrease in VTA DA neuronal activity after acute administration and withdrawal, respectively (for review, see Melis et al. 2005 and references therein).

One of the more fundamental, underlying, principles of addiction is that it is the result of the interaction of the drugs themselves in vulnerable individuals with genetic, environmental, behavioral, as well as other factors, which causes long-lasting adaptive changes in the function of the reward system. The individual vulnerability to drug addiction might be due to genetic make up, resulting in biological differences. However, theories about genetic basis of addiction other than alcoholism have been lacking because of the little information about the potential genetic contributions to vulnerability to psychostimulants and opiate addiction. Conversely, twin and adoption studies, studies of certain human populations (e.g., Japanese, Chinese), and animal models of alcoholism contributed to discover multiple genes contributing to this addiction (Enoch and Goldman 2001). In particular, some rat lines have been developed through selective breeding exhibiting high or low alcohol preference, respectively (for review, see Colombo et al. 2006 and references therein). Among these, Sardinian alcohol-preferring (sP) and -nonpreferring (sNP) rats have been developed in our laboratories in the early 1980s and may provide a model of how genetic factors contribute to the differential sensitivity to some behavioral actions of alcohol. Hence, sP rats meet most of the fundamental requirements posed when defining an animal model of alcoholism. In fact, when compared with sNP rats, they (1) ingest larger amounts of alcohol attaining psycho-pharmacologically relevant blood alcohol levels, (2) perform some degree of "work"—such as lever-responding—to gain access to alcohol, and (3) develop tolerance to a given effect of alcohol induced by their voluntarily consumption (see Colombo et al. 2006). Nonetheless, the neurobiological basis underlying the predisposition to alcohol preference and consumption of sP rats is still poorly understood. When compared with sNP rats, the sP rats exhibit higher levels of anxiety-related behaviors (Colombo et al. 2006) and lower density of dopaminergic fibers in the shell of the nucleus accumbens (Casu et al. 2002) where the density of DA $D_2$ (Stefanini et al. 1992) and $D_1$ (De Montis et al. 1993) receptors were found to be lower. In addition, in the shell of the nucleus accumbens of sP rats the extracellular levels of DA were higher when compared to sNP rats (Leggio et al. 2003). Altogether, this evidence suggests a difference in spontaneous activity of VTA DA neurons of sP rats when compared with sNP rats.

Despite the potential significance of a better understanding of the mechanisms underlying the neurobiological basis of alcohol preference and avoidance, several key pieces of information on neuronal activity of VTA DA cells in sP and sNP rats are yet unknown. Primarily, is VTA DA neuronal spontaneous activity similar in sP and sNP rats? Are there any differences in properties of afferent synapses onto VTA DA cells? Are endocannabinoids and CB1 receptors involved in the innate predisposition and avoidance of sP and sNP rats, respectively?

Thus, we examined the electrophysiological properties of VTA DA neurons of sP and sNP rats, both in vivo and in vitro, in order to elucidate the neurobiological basis of their alcohol preference and avoidance, respectively.

## Materials and methods

All experiments followed international guidelines on the ethical use of animals from the European Communities Council (EEC) (86/609/EEC).

### In vivo recordings

Male sP and sNP rats (250–350 g), from the 69th generation, were anesthetized with chloral hydrate (400 mg/kg, i.p.), their femoral vein was cannulated for i.v. administration of pharmacological agents, and they were placed in the stereotaxic apparatus (Kopf, Tujunga, CA, USA) with their body temperature maintained at 37±1°C by a heating pad. Thereafter, the scalp was retracted and one burr hole was drilled above the VTA (AP, 2.0 mm from lambda; *L*, 0.3–0.6 mm from midline) for the placement of a recording electrode. Single-unit activity of neurons located in the VTA (V, 7.0–8.0 mm from the cortical surface) was recorded extracellularly with glass micropipettes filled with 2% pontamine sky blue dissolved in 0.5 M sodium acetate (impedance, 2–5 MΩ). Single-unit activity was filtered (bandpass, 500–5,000 Hz) and individual spikes were isolated by means of a window discriminator (Digitimer, Hertfordshire, UK) and displayed on a digital storage oscilloscope (TDS 3012, Tektronics, Marlow, UK). Experiments were sampled on-line and off-line with the Spike2 software (Cambridge Electronic Design, Cambridge, UK) by

a computer connected to CED 1401 interface (Cambridge Electronic Design, Cambridge, UK). Single units were isolated and identified according to the already published criteria (Grace and Bunney 1983; Ungless et al. 2004). VTA DA neurons were selected when all criteria for identification were fulfilled: firing rate, <10 Hz; duration of action potential, >2.5 ms as measured from start to end, inhibitory responses to hindpaw pinching (Ungless et al. 2004). Bursts were defined as the occurrence of two spikes at an interspike interval of <80 ms and terminated when the interspike interval exceeded 160 ms (Grace and Bunney 1983).

### In vitro recordings

The preparation of VTA slices was as described previously (Bonci and Malenka 1999). Briefly, male sP and sNP rats (14–34 days), from the 69th generation, were anesthetized with halothane and euthanized. A block of tissue containing the midbrain was rapidly dissected and sliced in the horizontal plane (300 µm) with a vibratome (Leica) in ice-cold low-$Ca^{2+}$ solution containing (in millimolar): 126 NaCl, 1.6 KCl, 1.2 $NaH_2PO_4$, 1.2 $MgCl_2$, 0.625 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose. Slices were transferred to a holding chamber with artificial cerebrospinal fluid (ACSF, 37°C) saturated with 95% $O_2$ and 5% $CO_2$ containing (in millimolar): 126 NaCl, 1.6 KCl, 1.2 $NaH_2PO_4$, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose. Slices were allowed to recover for at least 1 h before being placed, as hemislices, in the recording chamber and superfused with the ACSF (34–36°C) saturated with 95% $O_2$ and 5% $CO_2$. Cells were visualized with an upright microscope with infrared illumination (Axioskop FS 2 plus, Zeiss), and whole-cell patch-clamp recordings were made by using an Axopatch 200B amplifier (Axon Instruments, CA, USA). Current-clamp experiments and inhibitory postsynaptic currents (IPSCs) recordings were made with electrodes filled with a solution containing the following (in millimolar): 144 KCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3.45 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 1 $CaCl_2$, 2.5 $Mg_2$ATP, and 0.25 $Mg_2$GTP (pH 7.2–7.4, 275–285 mOsm). All excitatory postsynaptic currents (EPSC) recordings were made with electrodes filled with a solution containing the following (in millimolar): 117 Cs methanesulfonic acid, 20 HEPES, 0.4 ethylene glycol bis (2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2.8 NaCl, 5 TEA–Cl, 2.5 $Mg_2$ATP, and 0.25 $Mg_2$GTP (pH 7.2–7.4, 275–285 mOsm). Experiments were begun only after series resistance had stabilized (typically 15–40 MΩ). Series and input resistance were monitored continuously on-line with a 5-mV depolarizing step (25 ms). Data were filtered at 2 kHz, digitized at 10 kHz, and collected on-line with acquisition software (pClamp 8.2, Axon Instruments, CA, USA). Dopamine neurons from the posterior VTA were identified by the presence of a large $I_h$ current (109±22.4 pA, $n$=23; Fig. 3b) (Johnson and North 1992) that was assayed immediately after break-in, using a series of incremental 10-mV hyperpolarizing steps from a holding potential of −70 mV. Dopamine cells with long-duration action potentials >3 ms (measured from the action potential threshold to the maximum after-hyperpolarization period) responded to DA (30 µM, 5 min) with a hyperpolarization (10.4±0.4 mV, $n$=23, data not shown). A bipolar stainless steel stimulating electrode (FHC, USA) was placed 100 µm rostral to the recording electrode and was used to stimulate at a frequency of 0.1 Hz. Paired stimuli were given with an interstimulus interval of 50 ms, and the ratio between the second and the first PSCs was calculated and averaged for a 5-min baseline. The depolarizing pulse used to evoke depolarization-induced suppression of inhibition (DSI) was a 500-ms to 10-s step to +40 mV from holding potential (−70 mV). The magnitude of DSI was measured as the percentage of the mean amplitude of consecutive IPSCs after depolarization (acquired between 5 and 15 s after the end of the pulse) relative to that of five IPSCs before the depolarization (Melis et al. 2004). The spontaneous miniature IPSCs (mIPSC) were collected in the presence of lidocaine (500 µM) and analyzed using the Mini Analysis program (Synaptosoft). To accurately determine the mIPSC amplitude, only mIPSCs that were >10 pA were accepted for analysis. All the numerical data are given as the mean±SEM and compared using the Student's $t$ test. Each slice received only a single drug exposure. Drugs were applied in known concentrations to the superfusion medium. All the drugs were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was <0.01%. Averaged data from different experiments are presented as the mean±SEM. Statistical significance was assessed using one- or two-way analysis of variance (ANOVA) for repeated measures followed either by Dunnett's or $t$ test where appropriate.

## Results

### VTA DA neuronal activity of sP rats differs from sNP rats in vivo

Extracellular single-unit recordings were made from eight sP and 11 sNP rats in which the recording electrode was histologically verified as being in the posterior VTA (Fig. 1a). We chose this location within the VTA because most of the nucleus accumbens-projecting DA neurons are mainly located in this quadrant of the VTA (Ford et al. 2006) and also because it has been shown to be the neuroanatomical substrate mediating the reinforcing effects of alcohol in rats (Rodd-Henricks et al. 2000; Rodd et al. 2005a, b; Rodd et al. 2004; Rodd et al. 2005c). Only DA

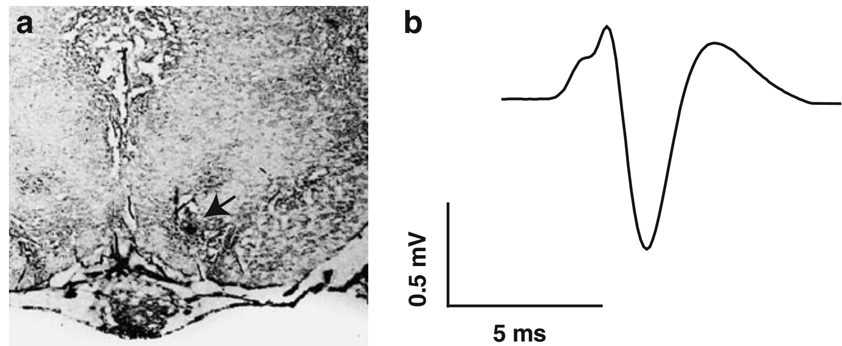



**Fig. 1** Identification of VTA DA neurons in vivo. **a** Photograph of a representative location of a recording site in the VTA. The *arrow* indicates the pontamine sky blue dye. **b** Average trace, acquired from a digital storage oscilloscope, showing the typical, broad, notched waveform of a VTA DA neuron recorded from an anesthetized sP rat

cells meeting the electrophysiological criteria for DA neurons were included in this study. Action potentials recorded from both sP and sNP rats displayed a typical, broad, notched waveform as shown in Fig. 1b. In agreement with studies carried out in other lines of rats selectively bred for their alcohol preference/avoidance (e.g., Indiana alcohol-preferring [P] and -nonpreferring [NP] rats; Morzorati 1998; Morzorati and Marunde 2006), an analysis of the number of spontaneously active VTA DA neurons per electrode track for sP and sNP rats did not reveal any difference between the two lines, since the mean (±SEM) number of DA neurons encountered in the VTA was 0.8±0.2 and 0.7±0.1 cells per track, respectively. Interestingly, the average spontaneous firing rate of VTA DA cells of sP and sNP rats was different, being 4.26±0.29 ($n$=51) and 3.61±0.18 Hz ($n$=56), respectively (Fig. 2; $t$ test: $t$=1.97; $P$<0.05). However, when the burst parameters were analyzed (i.e., percent of spikes in burst, burst rate, mean spikes per burst, mean burst duration, and mean intraburst frequency) no difference was revealed between sP and sNP rats (Table 1).

## Comparison of VTA DA neurons from sP and sNP rats in vitro

Whole-cell patch-clamp recordings were made from medial posterior VTA DA neurons in rat horizontal slices containing the midbrain. VTA DA cells of sP and sNP rats displayed similar electrophysiological characteristics, which, apart from their location in the slice, facilitated their identification (Grace and Onn 1989). Membrane properties of VTA DA cells were assessed immediately after break-in to avoid intracellular dialysis of the cell over time (Schaap et al. 1999). An analysis of the intrinsic properties of VTA DA cells did not reveal any difference between sP and sNP rats. In particular, VTA DA cells fired spontaneously at a regular rate (sP=2.27±0.26 Hz, $n$=21; sNP=2.26±0.18 Hz, $n$=21; two-tailed unpaired $t$ test, $P$>0.05; Fig. 3a) with long-duration action potentials (Fig. 3a), a mean input resistance (sP=192±16 MΩ, $n$=7; sNP=188±14 MΩ, $n$=9; $P$>0.05), and showed a hyperpolarization-activated inward-current ($I_h$), that were similar between sP and sNP rats (sP=125.1±23.72 pA, $n$=17; sNP=115.0±26.25 pA, $n$=17; two-tailed unpaired $t$ test, $P$>0.05; Fig. 3b–c).

## sP rats have a reduced probability of GABA release in the VTA

The discrepancy between the in vivo and in vitro results unveils the importance of the afferent inputs on the control of the spontaneous activity of VTA DA neurons (Johnson and North 1992). Therefore, to investigate whether changes at the synaptic level might underlie the differences observed in the spontaneous activity of sP and sNP rats in vivo, we studied postsynaptic excitatory- and inhibitory-mediated currents (i.e., EPSCs and IPSCs). Because an important factor controlling VTA DA cell firing is the glutamatergic input from cortical and subcortical regions (Geisler et al. 2007), which forms excitatory synapses on VTA DA cells (Christie et al. 1985; Sesack and Pickel 1992), in the first set of experiments, we compared the property of the excitatory synapses on VTA DA cells of sP and sNP rats. Figure 4a shows that the change in synaptic strength elicited by paired stimuli given at an interval of 50 ms was similar in sP and sNP rats. In fact, excitatory synapses on VTA DA cells of both groups exhibited a similar paired-pulse facilitation (sP: $s_2/s_1$ ratio=1.31±0.09; sNP=1.26±0.12; $n$=5 for both groups; two-tailed unpaired $t$ test, $P$>0.05). In contrast, GABAergic synapses were found to be different in sP rats when compared with sNP rats. In fact, sP rat VTA DA cells exhibited a larger paired-pulse facilitation when compared with sNP rats (sP: $s_2/s_1$ ratio=1.28±0.04; sNP=0.92±0.03; $n$=6 for both groups; $t$=6.318; two-tailed unpaired $t$ test, $P$<0.0001; Fig. 4b), which did not depend on the size of the first $GABA_A$ IPSC (IPSC1; Fig. 4c).

It has been shown that changes in the paired-pulse ratio might reflect either changes in the probability of GABA release (Bonci and Williams 1997; Melis et al. 2002) or function of postsynaptic $GABA_A$ receptors, or a combination of these. To determine whether changes in $GABA_A$ receptor function, number, or both occur in sP rats, we examined spontaneous miniature $GABA_A$ IPSCs (i.e., mISPCs).

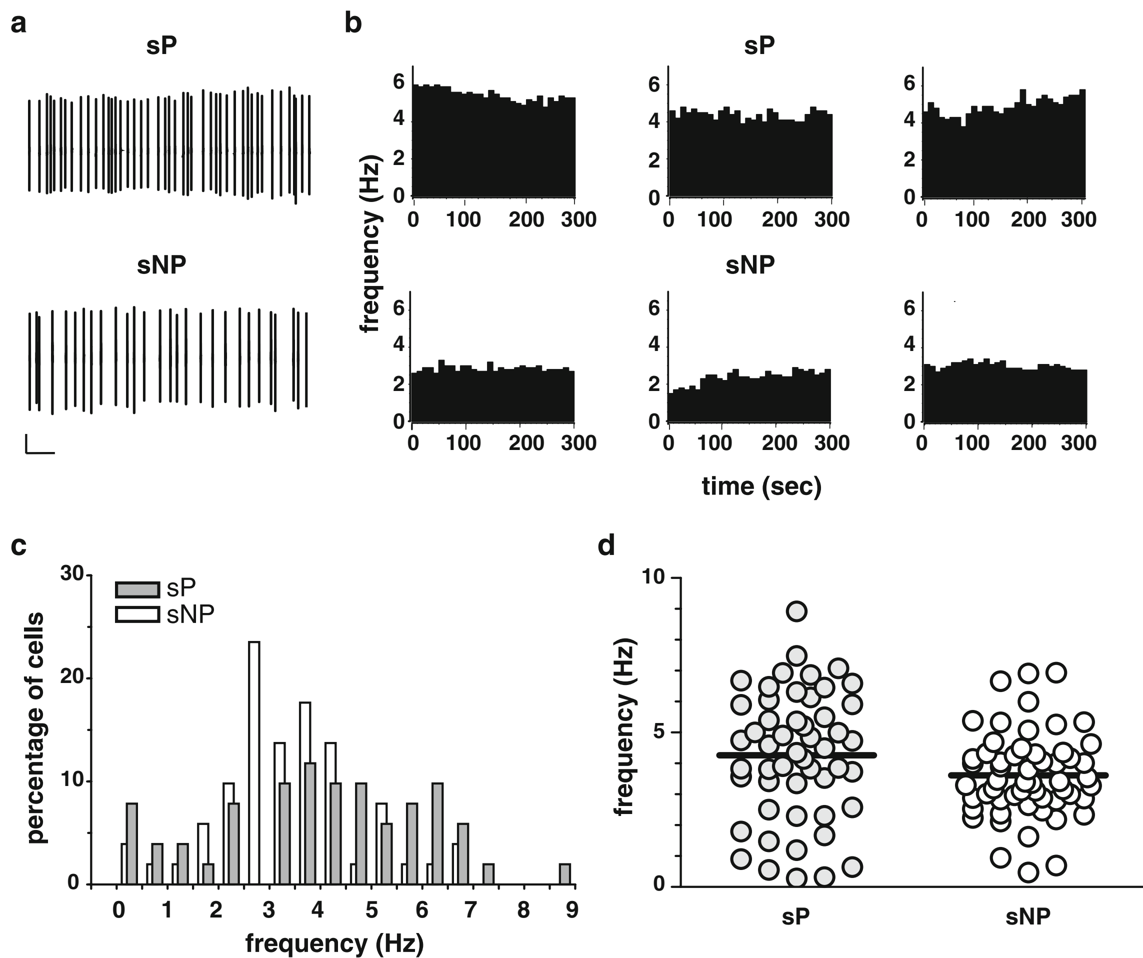



**Fig. 2** Comparison of VTA DA cells from alcohol-naïve sP and sNP rats in vivo. **a** Representative firing traces of posterior VTA DA neurons of sP (*top*) and sNP (*bottom*) rats. Scale bar=100 mV, 1 s. **b** Spontaneous activity of VTA DA neurons encountered in sP (*top*) and sNP (*bottom*) rats. Each histogram represents the spontaneous activity of a single DA neuron. Note the difference in spontaneous activity in the two lines. **c** Frequency distribution of spontaneous firing rate of VTA DA cells. Note the difference in frequency distribution of VTA DA cells belonging to sP and sNP rats. **d** Individual firing rates of VTA DA neurons recorded from sP and sNP rats. Each *circle* represents the mean firing rate of 5-min recording. *Horizontal bars* indicate the mean activity

Figure 5a–c shows that the frequency of mIPSCs was significantly lower in sP than in sNP rats (sP=0.1±0.004 Hz, $n$=10; sNP=0.9±0.1 Hz, $n$=8; two-tailed unpaired $t$ test, $P<0.00001$). Furthermore, there was no significant difference in the amplitude of mIPSCs with mean amplitudes of 24.1±1 and 24.7±7.3 pA in sP and sNP rats, respectively (Fig. 5a,d; two-tailed unpaired $t$ test, $P>0.5$). Because a decrease in frequency but not amplitude of mIPSCs is generally thought to reflect a presynaptic decrease in the probability of transmitter release (Katz 1971), both the

**Table 1** Basal characteristics of VTA DA neurons in sP and sNP rats in vivo

| | sP | sNP | |
|---|---|---|---|
| Frequency (Hz) | 4.3±0.3 ($n$=51) | 3.6±0.2 ($n$=56) | *$P<0.05$ |
| Spike duration (ms) | 6.3±0.2 ($n$=51) | 6.2±0.1 ($n$=56) | NS |
| Var. coeff. | 53.8±3.6 ($n$=51) | 61.8±4.7 ($n$=56) | NS |
| Percentage of spikes in bursts | 16.6±2.9 ($n$=51) | 16.7±3.3 ($n$=56) | NS |
| Mean spikes per burst | 3.3±0.2 ($n$=47) | 2.9±0.1 ($n$=49) | NS |
| Burst rate (Hz) | 0.2±0.04 ($n$=47) | 0.2±0.04 ($n$=49) | NS |
| Mean intraburst frequency (Hz) | 28.3±1.7 ($n$=47) | 28.9±2.3 ($n$=49) | NS |
| Mean burst duration (ms) | 186.9±20.8 ($n$=47) | 145.8±10.7 ($n$=49) | NS |

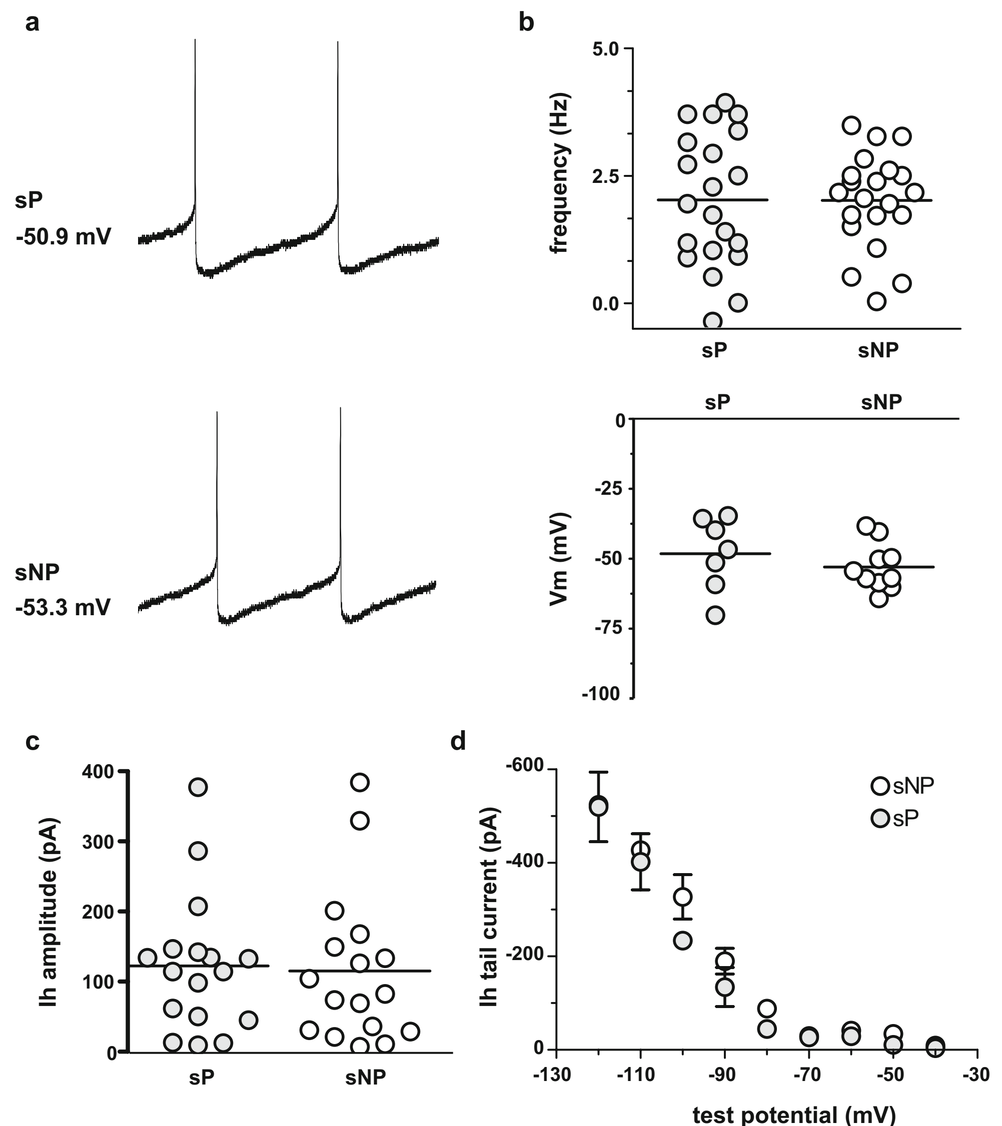



**Fig. 3** Comparison of VTA DA cells from alcohol-naïve sP and sNP rats in vitro. **a** Representative traces of action potential firing in sP (*top*) and sNP (*bottom*) rats. **b** Individual firing rates of VTA DA neurons recorded from sP and sNP rats. Each *circle* represents the mean firing rate of 3-min recording. *Horizontal bars* indicate the mean activity. **c** Individual voltage membrane (*Vm*) of VTA DA neurons recorded from sP and sNP rats. Each *circle* represents the mean Vm of 3-min recording. *Horizontal bars* indicate the mean Vm. **d** Tail current amplitudes, after subtraction of the current amplitude after no hyperpolarizing voltage step, are plotted versus the preceding test potentials

paired-pulse protocol and the decreased frequency of spontaneous miniature events indicate that the probability of GABA release in the VTA was decreased in sP rats.

## Difference in endocannabinoid-mediated transmission between sP and sNP rats

Converging evidence suggests a link between an altered endocannabinoid system and a genetic vulnerability to alcohol abuse (Colombo et al. 2005). Particularly, we found that the endocannabinoid system participates in the electrophysiological actions of alcohol on neuronal activity in the limbic system (Perra et al. 2005). Therefore, to investigate whether the decreased probability of GABA release in the VTA observed in sP rats includes changes in the endocannabinoid system, we compared an endocannabinoid-mediated form of short-term plasticity occurring at GABAergic synapses, which is DSI (Wilson and Nicoll 2001), in sP and sNP rats. This original mode of action for endocannabinoids allows their retrograde signal to decrease neurotransmitter release via the activation of CB1 receptors and to regulate synaptic transmission in many brain regions (Lovinger 2008). As shown in Fig. 6a, 5 and 10 s depolarization induced a significant DSI in sP rats (IPSCs amplitude after the depolarizing pulse was 61.22±4.1% and 52.75±4.5% of baseline for 5 and 10 s, respectively; $n$=6; paired $t$ test, $P$<0.0005 for both durations of depolarization). Remarkably, DSI was dramatically smaller in sP rats when compared with the one induced in sNP rats (two-way ANOVA, $F$(alcohol preference)$_{1,40}$=34.07, $P$<0.0001; $F$(duration of depolarization)$_{3,40}$=9.78, $P$<0.0001; Fig. 6a) where even a 1-s depolarization induced a significant DSI (IPSCs amplitude after the depolarizing pulse was 64.76±5.9% of baseline; $n$=6; paired $t$ test, $P$<0.003). Importantly, the different extent of DSI did not depend on the size of the first

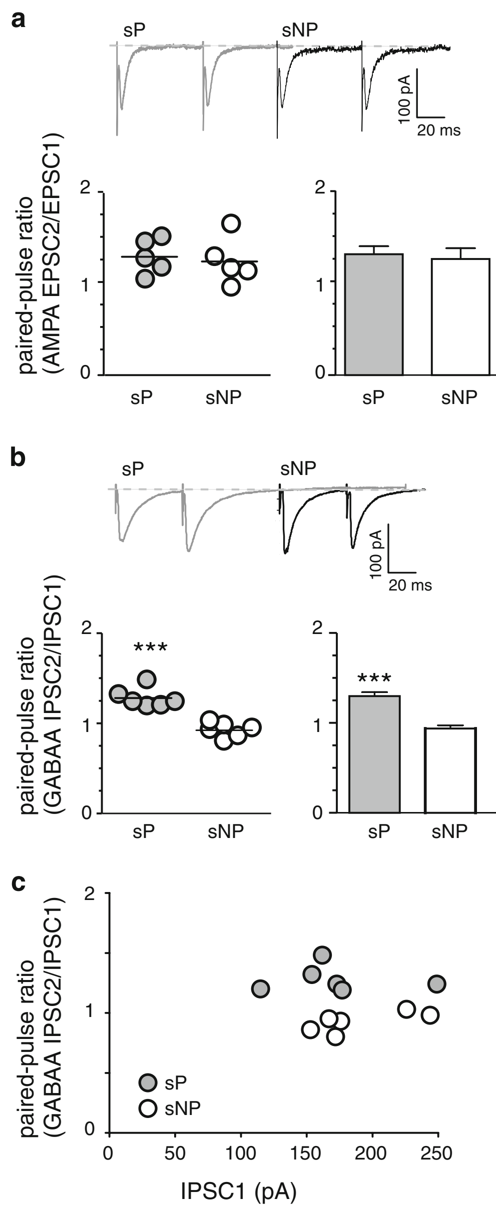



**Fig. 4** Alcohol-naïve sP rats show a reduced probability of GABA release in the VTA. **a** Paired-pulse ratio of AMPA EPSCs is similar in sP and sNP rats. *Left panel* individual AMPA-mediated EPSCs from VTA DA neurons recorded from sP and sNP rats. Each *circle* represents the mean paired-pulse ratio of 2-min recording. *Horizontal bars* indicate the mean paired-pulse ratio for all the cells recorded. *Right panel* bar graph summarizing the mean paired-pulse ratio of AMPA EPSCs for all cells recorded in sP and sNP rats. Examples of recordings are shown in the *insets*. **b** $GABA_A$ IPSCs of sP rats show larger paired-pulse facilitation compared to sNP rats ($P<0.0001$). *Left panel* individual $GABA_A$ IPSCs from VTA DA neurons recorded from sP and sNP rats. Each *circle* represents the mean paired-pulse ratio of 2-min recording. *Horizontal bars* indicate the mean paired-pulse ratio for all the cells recorded. *Right panel* bar graph summarizing the average paired-pulse ratio of $GABA_A$ IPSCs for all cells recorded in sP and sNP rats. Examples of recordings are shown in the *insets*. **c** No correlation was found between the amplitude of IPSC1 and the IPSC2/IPSC1 ratio in both sP and sNP rats

$GABA_A$ IPSC (IPSC1; Fig. 4c), being similar between sP and sNP rats.

The different magnitude of DSI in sP and sNP rats might reflect either a lower level of endocannabinoids and/or a reduced number or function of CB1 receptors. To assess whether differences in CB1 receptor function/number occur at GABAergic synapses in VTA DA neurons of sP rats, the synthetic CB1 receptor agonist WIN55,212-2 (WIN) was applied in a separate group of experiments. Bath application of WIN (0.01–3 μM, 5 min) significantly reduced $GABA_A$ IPSCs at the doses of 1 and 3 μM (by 30±4% and 55±7%, respectively; $n$=5; one-way ANOVA+Dunnett's test, $F_{5,25}$= 7.885, $P<0.0001$; Fig. 6c). Remarkably, when compared to sNP rats, the effect of WIN on $GABA_A$ IPSCs was considerably smaller (two-way ANOVA, $F$(alcohol preference)$_{1,34}$=63.11, $P<0.001$; $F$(dose)$_{4,34}$=59.68, $P<0.0001$; $F$(interaction)$_{4,34}$=6.76, $P$=0.0004; Fig. 6c), being significant at the dose of 0.3 μM (by 52±2% of baseline; $n$=5; one-way ANOVA+Dunnett's test, $F_{5,20}$=172.4, $P<0.0001$; Fig. 6c).

## Discussion

In the present study, we observe that posterior VTA DA neurons of alcohol-naïve sP rats in vivo fire more frequently than those of sNP rats. This phenomenon is accompanied by a decreased GABAergic transmission on VTA DA cells and an impaired endocannabinoid system. We hypothesize that this type of plasticity may contribute to alcohol preference in alcohol-naïve sP rats.

The observation that VTA DA cells of alcohol-naïve sP rats show an increased firing rate is consistent with and extend the notion that individual vulnerability to drug-seeking behavior is associated with increased impulse activity of DA cells (Marinelli et al. 2003; Marinelli et al. 2006; Marinelli and White 2000), whereas a decreased DA cell activity is associated with resistance to the development of addictive behaviors (Brandon et al. 2001; Marinelli et al. 2003; Marinelli and White 2000). Accordingly, the increased DA cell firing activity of sP rats can explain the higher extracellular DA levels detected in their shell of the nucleus accumbens (Leggio et al. 2003). Additionally, other studies report that VTA DA neurons of either Indiana alcohol-preferring P or Lewis rats burst-fired more frequently than the Indiana alcohol-nonpreferring NP line and Fisher344 rats, respectively (Minabe et al. 1995; Morzorati 1998; Morzorati and Marunde 2006). Notably, we found that bursting activity (indexed by both percentage of spikes in burst and burst rate) was lower in these lines of rats, when compared with other outbred rat strains (e.g., Sprague–Dawley, Wistar rats). We do not have a likely explanation for this finding; however, any comparison with other strains is difficult to interpret, since sP and sNP may

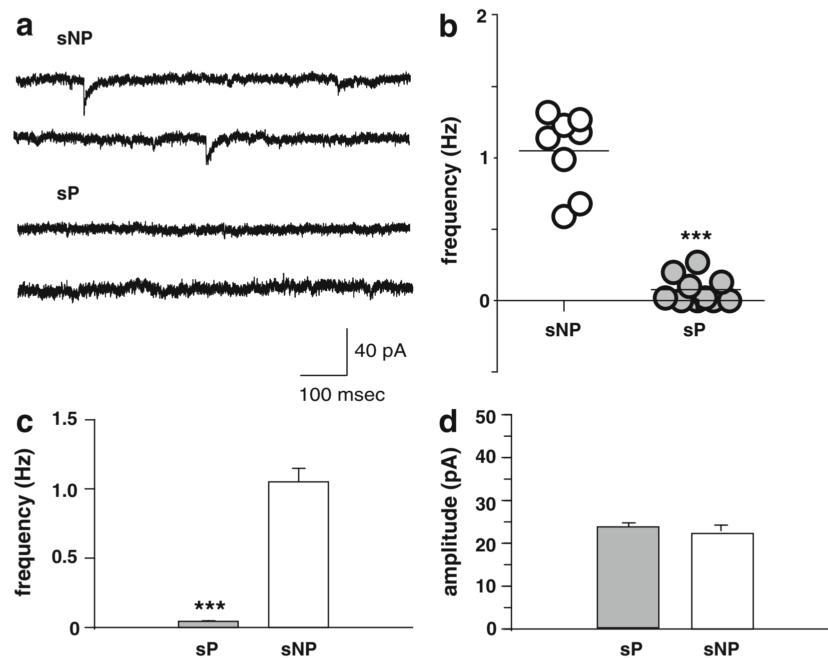
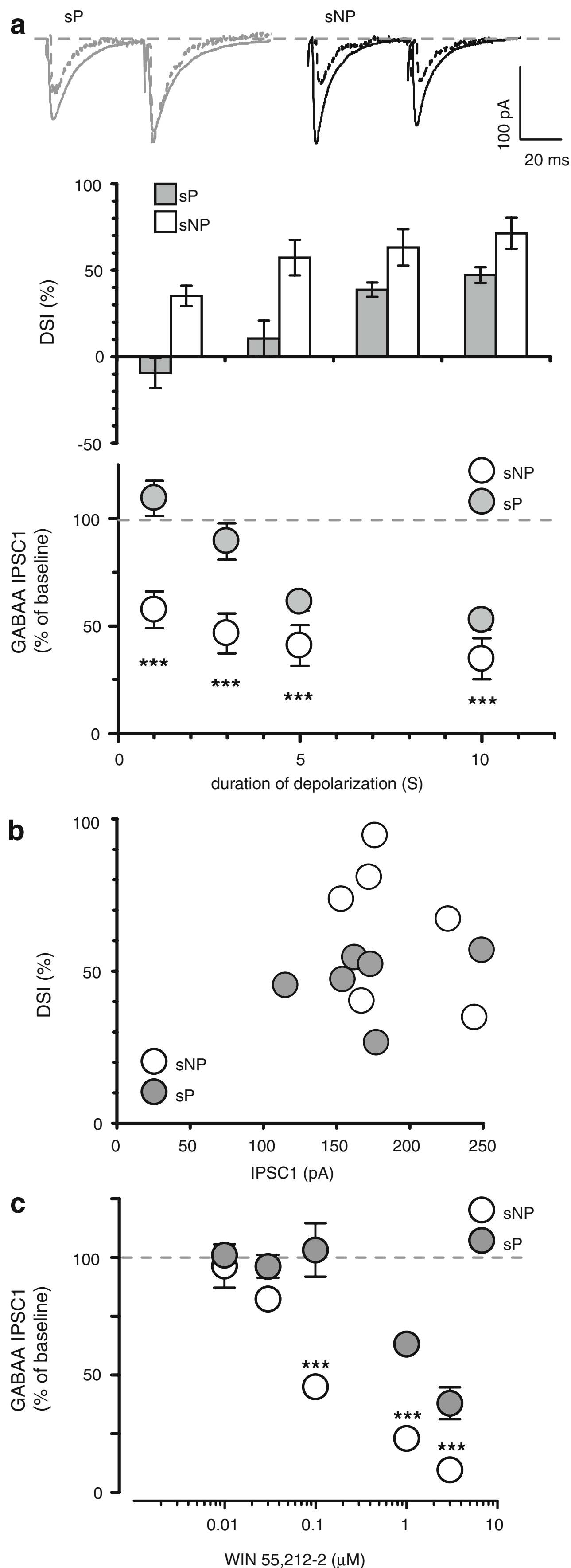



**Fig. 5** Alcohol-naïve sP rats have a decreased frequency, but not amplitude, of GABAA mIPSCs. a Samples of mIPSCs from naïve sP (top traces) and sNP (bottom traces) rats. b Individual frequency of mIPSCs recorded from naïve sP and sNP rats. Each circle represents the mean frequency of 2-min recording. Horizontal bars indicate the mean activity (P<0.0001). c Bar graph showing the average (mean±SEM) frequency for sP and sNP rats (*P*<0.0001). **d** Bar graph showing the average (mean±SEM) amplitude for mIPSCs from sP and sNP rats

have developed a specific genetic background across generations, which may influence several phenotypic features, including regulation of firing pattern. Nevertheless, our findings support the hypothesis that a higher VTA DA neuronal spontaneous activity, either in terms of firing rate or pattern, is associated with alcohol preference in rats and, ultimately, with increased vulnerability to alcohol.

Although the exact underlying mechanism is uncertain, the neuroadaptations occurring at GABAergic synapses onto VTA DA cells of alcohol-naïve sP rats, which we observed in vitro, might contribute significantly to the increased impulse activity of DA cells. Indeed, the second set of experiments revealed that, in sP rats the paired-pulse modulation of $GABA_A$ IPSCs showed a larger facilitation. The paired-pulse stimulation is typically used as an electrophysiological protocol to test for changes in the probability of transmitter release (see Melis et al. 2002 and references therein). However, a decrease in the probability of GABA release might be simply one of many factors determining the larger facilitation observed in sP rats. Although a postsynaptic mechanism could account for this phenomenon, we tend to rule out this possibility because sP rats also show a dramatic decrease in mISPCs frequency, but not in amplitude, when compared with sNP rats. Thus, the decreased $GABA_A$-mediated inhibition may be considered a measure of changes occurring at these synapses, eventually leading to an increased DA cell firing activity and contributing to the expression of alcohol preference.

An attractive explanation for the observed larger facilitation in sP rats would have been a larger number of presynaptic CB1 receptors or a bigger effect produced by their activation. Unexpectedly, our results indicate that CB1 receptors might be either less functional or fewer in number. Indeed, we found that both DSI and the CB1 receptor agonist WIN55,212-2 produced a smaller inhibition of $GABA_A$ IPSCs in alcohol-naïve sP rats when compared with sNP rats. However, since CB1 receptor activation acts as a negative feedback mechanism to regulate GABAergic transmission within the VTA, the observed reduction might be the consequence of a compensatory mechanism to maintain sufficient GABA levels within the VTA. Whether this reduced endocannabinoid signaling results in an altered cAMP–PKA pathway modulating GABA release has yet to be elucidated. Nonetheless, it is important to note that changes in the endocannabinoid system have been implicated in neural plasticity associated with alcohol preference and dependence (Basavarajappa 2007; Basavarajappa et al. 1998; Basavarajappa and Hungund 2002; Basavarajappa et al. 2000; Colombo et al. 2005; Hungund et al. 2002; Hungund et al. 2003; Mechoulam and Parker 2003; Wang et al. 2003). Indeed, in agreement with our findings, innate predisposition to drink alcohol has been reported to be associated with a significantly lower level of CB1 receptor binding sites and higher affinity for the CB1 receptor agonist [$^3$H]CP-55,940 in alcohol-preferring




◀ **Fig. 6** Endocannabinoid modulation of inhibitory transmission is impaired in alcohol-naïve sP rats. **a** Averaged data for DSI induced by depolarizing pulses with a duration of 1, 3, 5, and 10 s are plotted in the *upper panel*. The relationship between the depolarizing pulse duration and the relative amplitude of $GABA_A$ IPSCs obtained after 5–15 s after the end of depolarization is plotted in the *lower panel*. $GABA_A$ IPSCs amplitude was normalized to the averaged value (*dotted line*) before depolarization. Each symbol represents the averaged value obtained from different cells ($n$=6; $P$<0.0001). Representative traces before and after 10 s DSI are overlaid and from a single experiment are shown (*top traces*). *Dotted traces* represent the $GABA_A$ IPSCs after the depolarizing step. **b** No correlation was found between DSI magnitude and size of GABAA IPSC. **c** Dose–response curves for percentage inhibition in GABAA IPSC size from VTA DA cells of sP and sNP rats ($n$=5; $P$<0.0001) produced by the CB1 receptor agonist WIN55,212-2

C57BL/6 mice (Basavarajappa and Hungund 2001; Hungund and Basavarajappa 2000). Conversely, in alcohol-avoiding DBA/2 mice, CB1 receptors are more numerous, although less often coupled to G proteins, when compared to C57BL/6 mice (Basavarajappa and Hungund 2001; Hungund and Basavarajappa 2000; Ortiz et al. 2004). In addition, brains of Fawn hooded rats (that exhibit high alcohol preference and consumption) display both lower regional levels of CB1 receptor function and gene expression when compared with Wistar rats (that may be considered as alcohol-avoiding rats) (Ortiz et al. 2004). Accordingly, our observations suggest that an impaired endocannabinoid system might contribute to neuroadaptative forms which could render individuals more susceptible to the pleasurable effects of alcohol and other drugs of abuse and/or eventually trigger drug-seeking behavior. The nature of these adaptive changes is currently not known and may be due to an increased endocannabinoid tone resulting in a compensatory downregulation of CB1 receptors. Whether these neuro-adaptations in alcohol-naïve sP rats are the result of a compensation for the effects of alcohol exposure, which would be transmitted over several generations, remains to be investigated. Remarkably, this would be consistent with studies reporting that both short- and long-term exposure to alcohol increases endocannabinoid levels in many brain regions (Basavarajappa and Hungund 1999; Basavarajappa et al. 2000; Gonzalez et al. 2002; Rubio et al. 2007; Vinod et al. 2006) including the midbrain (Gonzalez et al. 2004). Thus, the endocannabinoid-dependent plasticity occurring at these synapses might represent an important cellular signaling event underlying increased alcohol preference. It is worth mentioning, however, that although alcoholism runs in families and is partly inherited (Cadoret et al. 1995; Vaillant 1984), no one is born an addict. In fact, several genetic and environmental factors can add up or cancel each other out (Ellenbroek et al. 2005; Poikolainen 2000). More importantly, since it is not known what exactly is inherited (Gordis 1996), our results provide evidence that a genetic predisposition for alcohol is

associated to an increased spontaneous activity of DA neurons, together with both reduced endocannabinoid-mediated transmission onto GABAergic synapses and GABA release in the VTA. Follow-up studies are needed to examine the intracellular pathways involved in these synaptic changes in order to begin to identify the multitude of genes shaping the complex neuronal signaling which makes these alcohol-naïve sP rats prefer alcohol. Finally, the insights from this animal model for high risk for alcoholism might pave the way for new medications in treating alcoholism and preventing relapse.

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