Pitx3-deficiency in mice affects cholinergic modulation of GABAergic synapses in the Nucleus Accumbens

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ABSTRACT

We investigated to what extent Pitx3-deficiency, causing hyperdopaminergic transmission in the nucleus accumbens microcircuitry may lead to developmental changes. First, spontaneous firing activity of cholinergic interneurons in the nucleus accumbens was recorded \textit{in vitro}. The firing patterns in the Pitx3-deficient mice were more variable and intrinsically different from those observed in wildtype mice. Next, to test whether the irregular firing patterns observed in mutant mice affected the endogenous nicotinic modulation of the GABAergic input of medium spiny neurons, we recorded spontaneous GABAergic inputs to these cells before and after the application of the nicotinic receptor blocker mecamylamine. Effects of mecamylamine were found in slices of either genotype, but in a rather inconsistent manner. Possibly this was due to heterogeneity in firing of nearby cholinergic interneurons. Hence, paired recordings of cholinergic interneurons and medium spiny neurons were performed to more precisely control the experimental conditions of the cholinergic modulation of GABAergic synaptic transmission. We found that controlling action potential firing in cholinergic neurons leads to a conditional increase in GABAergic input frequency in wildtype mice but not in Pitx3-deficient mice. We conclude that Pitx3-deficient mice have neural adaptations at the level of the nucleus accumbens microcircuitry that in turn may have behavioural consequences. It is discussed to what extent dopamine release in the nucleus accumbens may be a long term gating mechanism leading to alterations in cholinergic transmission in the nucleus accumbens, in line with previously reported neural adaptations found as consequences of repeated drug treatment in rodents.

Key words: action potential, cholinergic neuron, current clamp, GABAergic neuron, IPSC, microcircuitry, nicotinic receptor, voltage clamp.
INTRODUCTION

Repeated exposure to addictive drugs is thought to cause the gradual neuroadaptations underlying the long lasting nature of the effects of addictive drugs on motivational behaviour (Everitt et al. 2001). Repeated administration of drugs of abuse causes a persistent increase in dopamine release in the nucleus accumbens (NAc). We recently showed that repeated in vivo amphetamine as well as morphine treatment is associated with an increase in the strength of cholinergic modulation of GABAergic transmission within the NAc shell (de Rover et al. 2004, 2005). It is however unclear whether the increase in endogenous cholinergic tonus is directly caused by the repeated administration of drugs of abuse, or a secondary effect caused by the increase in dopamine release in the NAc.

An experimental animal model that may shed new light on this issue is the Pitx3-deficient (or aphakia) mouse mutant. Pitx3 is a homeodomain gene that is necessary for the development of Substantia Nigra pars compacta (SNc) dopaminergic neurons (Smidt et al. 2004a). In Pitx3-deficient mice most dopaminergic neurons in the SNc are lost during development and consequently there is no dopamine release in the Caudate Putamen in these animals (Hwang et al. 2003; Nunes et al. 2003; Smidt et al. 2004a; Van den Munckhof et al. 2003). Therefore, Pitx3-deficient mice have been used as a genetic mouse model of Parkinsonism (e.g. Fleming et al. 2005). However, the behavioural phenotype of the Pitx3-deficient mice is rather mild and mainly motor output and not motor skills are affected (Smits et al. 2006). This suggests that adaptive changes in other brain regions may partly compensate for the loss of dopaminergic innervation of the Caudate Putamen in these mutant mice. In line with this a significantly higher percentage of the surviving dopaminergic neurons, mainly located in the ventral tegmental area (VTA) and innervating the NAc, was reported to be spontaneously active in Pitx3-deficient compared to wildtype mice (Smits et al. 2005). This indicates that there is more dopamine release in the NAc of Pitx3-deficient mice
compared to wildtype mice (Gonon and Buda 1985; Kuhr et al. 1987; Suaud-Chagny et al. 1992). Moreover, the gene expression pattern in the NAc of Pitx3-deficient mice (Smits et al. 2005) was found to be similar to the pattern found in DAT -/- mice, known to display a hyperdopaminergic tonus (Dumartin et al. 2000; Fauchey et al. 2000; Giros et al. 1996; Jones et al. 1998). Finally, Pitx3-deficient mice display increased climbing behaviour (Smidt et al. 2004a,b), which can also be induced by dopaminergic agonists (Costall et al. 1978). Together, these findings lead to the idea that Pitx3-deficient mice, like DAT -/- mice, have a constitutively increased dopaminergic tonus in their NAc (Smits et al. 2005), which may partly compensate for the loss of dopaminergic tonus in the Caudate Putamen. Understanding these adaptations may help to find better symptomatic treatments for Parkinson’s Disease.

We have recently reported that cholinergic neuromodulation of the GABAergic input onto medium spiny neurons, may play an important role in mediating downstream effects of alterations in the dopaminergic input from the VTA to the NAc (de Rover et al. 2002, 2004, 2005). In these previous reports, normal animals were conditioned with repeated in vivo injections with drugs of abuse, which was shown to lead to a conditional increase in the cholinergic modulation of the GABAergic transmission recorded in medium spiny neurons. Here we investigated whether the cholinergic transmission in the NAc of Pitx3-deficient mice was similarly affected.

MATERIALS AND METHODS

Mice

The Pitx3-deficient (Aphakia) strain (Rieger et al. 2001; Semina et al. 2000; Varnum and Stevens 1968) and C57Bl/6-Jico mice (Charles-River, The Netherlands) were used. All mice were bred at the Rudolf Magnus Institute for Neuroscience (Utrecht, The Netherlands) and
brought to the Vrije Universiteit Amsterdam (The Netherlands) on postnatal day 7. The animals were housed in Macrolon cages under controlled conditions (lights on from 2:00 p.m. to 2:00 a.m.). Standard food (Hope Farms, Woerden, The Netherlands) and tap water were available ad libitum. Upon arrival in the laboratory (Vrije Universiteit Amsterdam, The Netherlands) the animals were allowed to accustom to the housing facilities for at least 5 days before the beginning of the experiment. Experiments were done on mice ranging from 12 to 16 days PN. Experiments were done double blind; wildtype and Pitx3-deficient mice were taken from the nest by the experimenter without any prior knowledge about the genotypes. The heads were kept on ice and used for post-hoc determination of the genotypes. An expert determined the genotypes on the basis of the presence of a lens in the eyes of the animals, after analysis of the experiments.

Electrophysiology

After decapitation the brain was removed from the skull and placed for 3 minutes in ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl (125), KCl (3), NaH₂PO₄ (1.2), CaCl₂ (2.4), MgSO₄ (1.3), NaHCO₃ (25) and D-glucose (10) (pH 7.2). Transversal whole brain slices of 300 µm thickness were cut on a Leica vibratome (Nussloch, Germany). The parts containing the striata (dorsal and ventral parts were not separated) were then isolated from the rest of the slice and transferred to a storage chamber, filled with carboxygenated ACSF (5% CO₂, 95% O₂) stored on ice (4 °C).

Fifteen minutes prior to measurements, slices were transferred to the recording chamber, where they were continuously perfused with carboxygenated ACSF at 33°C. Only neurons in the NAc and preferably in the NAc shell were recorded (Paxinos and Franklin 2001). Although the transversal slices that we used provided the best opportunity to distinguish between the NAc core and shell regions using light microscopy, due to the age of the mice and thus the size of their NAc, we could not exclude the possibility that a small
minority of our recordings were made from NAc core neurons. Further, because of the size of the NAc of the mice that were used, no discrimination between different subregions within the NAc shell was made. Instead, we aimed at recording throughout the whole NAc shell. Neurons were identified by morphological as well as electrophysiological criteria as published previously (Kawaguchi et al. 1995). More specifically, voltage clamp recordings were made from GABAergic cells, which were identified by their small soma size and compact cell appearance (not elongated). Further, only a very little leakage current was tolerated at a holding potential of -70 mV indicating that the natural membrane potential was not more depolarized than -70 mV. Current clamp recordings were made from cholinergic interneurons which were identified by their large soma size, around 2x the average (GABAergic cell) soma size, spontaneous AP firing, typical AP shape, namely with a large (at least 10 mV) and relatively long-lasting undershoot. Voltage clamp recordings of GABAergic neurons were always performed at a holding potential of –70 mV. Only recordings in which the uncompensated series resistance was < 20 MΩ were analyzed. Series resistance was usually compensated by 70% and whole-cell capacitances averaged at 7 ± 3 pF, indicating that only GABAergic neurons were recorded (see further Result section for recording criteria). Pipettes for the voltage-clamp recordings (3-4 MΩ) were filled with (mM): CsCl (135.5), N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, 10), MgATP (2), ethylene glycol-bis (beta-aminoethyl ether) n,n,n’,n’-tetraacetic acid (EGTA, 11), Tris-GTP (0.1), CaCl₂ (1) (pH 7.2 with CsOH). Cesium was used to block potassium channels in order to increase the signal to noise ratio in these recordings and a high intracellular chloride concentration was used to set the chloride reversal potential at 0 mV to obtain a linear voltage-current relationship for the chloride-gating GABAₐ receptors.

In contrast, in current clamp recordings, the intracellular solution contained a low concentration of chloride in order to establish a more natural chloride reversal potential. Gluconate was used as a replacement of chloride. Furthermore, potassium was used instead
of the potassium channel blocker cesium because functioning potassium channels are essential for the occurrence of action potentials. Electrodes for the current-clamp recordings of cholinergic (ACh) neurons were filled with (mM): K-glucuronate (121), KCl (9), HEPES (10), MgATP (4), phosphocreatine (10) and Tris-GTP (0.3) (pH 7.2 with KOH). We used the amplitude of the action potential (AP) as a measure of the quality of the recording. Only cells with constant AP amplitudes were analyzed (max. 10% variation). Mecamylamine was applied through bath perfusion.

HEPES was from Life Technologies (Breda, The Netherlands). NaCl, NaHCO3, KCl, D-glucose, MgATP, EGTA, Tris-GTP and K-glucuronate were from Sigma (St. Louis, MO, USA). 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) and mecamylamine were from RBI (Natick, MA, USA). NaH2PO4 was from Merck (Darmstadt, Germany). CaCl2 and MgSO4 were from Baker (Deventer, The Netherlands). CsCl was from ICN (Zoetermeer, The Netherlands) or Sigma.

Data acquisition and analysis of action potential firing

Data were stored on DAT, digitized at a sampling frequency of 10 kHz and analyzed offline by the Strathclyde software EDR and WCP (J. Dempster, University of Strathclyde, Glasgow, UK), using an amplitude-threshold criterion (i.e. ten times the standard deviation of the baseline noise) to detect action potentials.

The first 30 seconds of each recording were discarded because the firing frequency is usually high right after the transition from the cell-attached to the whole-cell mode. Lognormal functions were fitted to histograms of the binned interval data obtained from 300 action potentials per cell (starting at 30 seconds in the recordings). Lognormal or double lognormal fits were made using NLREG (non linear regression analysis, P.H. Sherrod, Brentwood, TN, USA) which results in average AP intervals (as tau values) with corresponding standard deviations depending on the peak width. The histograms were
compared by constructing cumulative probability plots followed by Kolmogorov-Smirnov testing (plots not shown, but results given in the Results section).

Data acquisition and analysis of synaptic transmission

Data were stored on DAT, digitized at a sampling frequency of 5 kHz and analyzed offline by the Strathclyde software EDR and WCP (J. Dempster, University of Strathclyde, Glasgow, UK), using an amplitude-threshold criterion (i.e. twice the standard deviation of the baseline noise) to detect synaptic events. We used the stability of the rise times to determine the quality of the recording. Only cells with constant rise times throughout the recording were analyzed. Quantitative analysis was performed as described in detail previously (Brussaard et al. 1996), i.e. frequency and amplitude of IPSCs were analyzed. Unimodal lognormal functions were fitted to histograms of binned amplitude data and unimodal exponential functions were fitted to histograms of binned interval data obtained from a minimum of 1000 IPSCs per histogram. The histograms were compared by constructing cumulative probability plots followed by Kolmogorov-Smirnov testing (plots not shown, but results given in the Result section and table 1). Further the results of the fits were compared with the Wilcoxon-matched pairs test of which the results are given in the Results section. Average effects mecamylamine (fig. 2) and action potentials (fig. 3) on amplitudes and frequencies of IPSCs were calculated by normalizing the results of the fits to the result under control conditions (100%) for each cell and averaging the normalized results of the fits per experimental condition (shown as bar graphs in fig. 2 and 3). As a measure of the variation under control conditions, the standard errors under control conditions were expressed as percentages of the average control values and visualized as horizontal bars in each bar graph (fig. 2 and 3).

Finally, it must be stated that data from fig. 2 and 3 were observed within individual recordings, in which case each time one successful recording was made per animal. Since, this design allows for pairwise statistical testing of experimental conditions within individual
recordings the significance is particularly strong. At the same time, by taking recordings from many different animals, the data are likely to be representative for the strain of mice, wildtype versus Pitx3-deficient, being tested.
RESULTS

Firing pattern of cholinergic interneurons.

First we recorded the spontaneous firing activity of cholinergic interneurons in current clamp. These neurons were identified based on the two-photon imaging of their morphology (de Rover et al. 2004) showing that their cell soma size (cell capacitance) and their spontaneous firing properties (Kawaguchi et al. 1995) are crucially different from GABAergic interneurons and/or medium spiny neurons. The ACh interneurons in slices of wildtype animals showed spontaneous, somewhat irregular firing over a wide range of frequencies of 0.7 – 13.5 Hz (n = 7). These 7 recordings were made in slices from 7 different wildtype mice and thus the range of frequencies may be regarded as representative for the firing of cholinergic neurons in the NAc of wildtype mice. The average firing frequency was found to be 9.4 ± 1.8 Hz, and two examples are shown in fig. 1A. The ACh interneurons in slices from Pitx3-deficient mice also showed a variety of firing frequencies between 1.6 – 24.6 Hz (n = 6). Again, this wide range of frequencies may be regarded as representative the firing of cholinergic neurons in the NAc of Pitx3-deficient mice since we recorded 6 different cells from 6 different Pitx3-deficient mice. The average firing frequency was found to be 9.0 ± 3.9 Hz, and three examples are shown in fig. 1B. The group-average of firing frequency did not differ significantly between the two groups of mice (Mann-Whitney; data not shown), however the range of firing frequencies that we measured appeared to be shifted for the mutant mice (1.6 – 24.6 Hz) compared to the wildtype mice (0.7 – 13.5 Hz). This may indicate a more variable action potential firing in the Pitx3-deficient mice. Hence, next we analyzed the firing patterns. For each recorded cell a histogram was made of at least 300 APs starting after 30 seconds of cell dialysis at the onset of the recording. Lognormal fits were made to these histograms, except in the cases a double lognormal fit resulted in a significantly better fit. In the case of the wildtype mice this procedure resulted in seven...
recordings, all of which were sufficiently described by single lognormal distributions, one with a $\tau$ of 1.36 sec and 6 with average $\tau$ values of around 0.1 sec (two example fits in fig. 1C and overview in fig. 1E). In the mutant mice, 4 unimodal lognormal fits and 2 bimodal lognormal fits were observed (example fits in fig. 1D and overview in fig. 1E). Moreover, the 4 unimodal recordings were variable, whereas the two remaining recordings had bimodal distributions. In contrast, in 6 out of 7 recordings in the wildtype mice the average firing frequency was around 0.1 Hz, and all (100%) of these distributions were fit best using an unimodal fit only. Together, the firing frequency and the firing pattern data suggested that action potential firing in the mutant mice is more variable and thus intrinsically different from that observed in wildtype mice (fig. 1C, D, E).

**Endogenous nicotinic modulation of GABAergic synapses.**

The spontaneous firing patterns observed in the putative cholinergic interneurons may result in endogenous release of ACh, possibly leading to nicotinic modulation of the GABAergic input of medium spiny neurons in the NAc shell, in line with previous observations in rats (de Rover et al. 2004). To test this hypothesis, we recorded spontaneous inhibitory postsynaptic currents (sIPSCs) from the medium spiny cells, before and after the application of mecamylamine, a nicotinic ACh receptor antagonist (1 $\mu$M). The GABAergic medium spiny neurons, which are the output neurons of this brain area, were identified by combined electrophysiological and morphological criteria, i.e. a two-photon laser scanning microscopy reconstruction was matched with electrophysiological properties as described previously (de Rover et al. 2004). Next, GABAergic medium spiny neurons were voltage clamped at –70 mV and sIPSCs were pharmacologically isolated from excitatory postsynaptic currents (EPSCs) using DNQX (20 $\mu$M). The mecamylamine effects on sIPSC frequencies were inconsistent (fig. 2). Overall, mecamylamine did not affect sIPSC frequency in either wildtype mice (fig. 2E) or mutant mice (fig. 2F). However, there was a considerable amount
of variation: in wildtype mice within individual recordings mecamylamine caused a substantial decrease in sIPSC frequency in 3 cells (p<0.01 KS), no effect in 3 other cells (p>0.05 KS) and an increase in sIPSC frequency in 2 cells (p<0.01 KS; table 1). The mecamylamine effect on sIPSC frequency was variable in mutant mice as well: mecamylamine caused a decrease in sIPSC frequency in 2 cells (p<0.01 KS), no effect in 2 other cells (p>0.05 KS) and an increase in sIPSC frequency in 3 cells (p<0.01 KS; table 1). In addition, the variation in the effect of mecamylamine on the sIPSC frequency was significantly larger in wildtype mice than in Pitx3-deficient mice (F-max test: p=0.035; note the error bar in fig. 2E1 versus the error bar in fig. 2E2).

Within individual recordings mecamylamine caused a significant decrease in sIPSC amplitude in 7 out of 8 cells in controls (fig. 2A, C, E2 and table 1, significant at p<0.01 in 5 cells and at p<0.05 in 2 other cells). This would imply the presence of a significant concentration of endogenous ACh in the slices of wildtype mice. The same holds true for 4 out of 7 cells recorded in slices from Pitx3-deficient mice, where we observed a similar suppression of the sIPSC amplitude upon application of mecamylamine (fig. 2B, D, F2 and table 1, significant at p<0.01 in 4 cells). In three additional recordings from mutant mice no changes were observed (table 1).

Evoked cholinergic modulation of GABAergic synapses

Since some effects of mecamylamine were observed in slices from both wildtype and Pitx3-deficient mice - be it in a rather inconsistent and apparent uncorrelated manner - these results were difficult to interpret. Possibly this was due to the fact that in these synaptic recording experiments, there were also differences as far as the variability in firing of nearby ACh interneurons is concerned (see fig. 1). Hence we performed paired recordings of ACh interneurons and medium spiny neurons to gain a better control over the experimental conditions of the cholinergic modulation of the GABA input in this type of experiment. To
this end, the ACh neurons were recorded in current clamp, while nearby GABAergic medium spiny neurons were recorded in voltage clamp. Initially, the ACh neurons were kept quiescent by hyperpolarizing current injection for a period of three minutes, during which so-called baseline sIPSCs were recorded (fig. 3A1, B1). Next, ACh neurons were allowed to fire at their endogenous frequency (no current injection) and again sIPSCs were recorded during AP firing in the ACh neurons (fig. 3A2, B2). The AP firing in ACh neurons in wildtype slices caused an increase in sIPSC frequency in 5 out of 7 recorded cell pairs (fig. 3A and C, overall average: 149.5 ± 27.8 % p<0.05), which was absent in the subsequent presence of mecamylamine (1 µM, data not shown). In contrast, in none of seven cell pairs in Pitx3-deficient mice did we observe any effect (fig. 3B and D, overall average: 103.9 ± 5.7 %, not significant).

There was no overall effect of ACh neuron firing on the sIPSC amplitudes: in wildtype mice the average sIPSC amplitude during AP firing in ACh neurons was 104.2 ± 16.2% compared to the average baseline sIPSC amplitude (not significant, KS). In Pitx3-deficient mice the average sIPSC amplitude during AP firing in ACh neurons was 94.5 ± 11.1% compared to the average baseline sIPSC amplitude (not significant, KS).

In conclusion, ACh released by spontaneous AP firing in ACh neurons causes an increase in sIPSC frequency without affecting the sIPSC amplitude and this effect is not present in Pitx3-deficient mice.
DISCUSSION

**Firing pattern of cholinergic interneurons.**

The first step in the circuit of cholinergic modulation of GABAergic synapses in the NAc is the neuronal activity of the cholinergic interneurons. The endogenous firing pattern of ACh interneurons was found to be different, although the average firing frequency was not significantly different. In wildtype mice we found only unimodal lognormal fits to the interval histograms, whereas in mutant mice the firing frequency appeared more variable. We previously showed that repeated *in vivo* amphetamine treatment in rats causes more variable firing patterns of ACh neurons in the NAc (de Rover et al. 2004). Bimodal (or burst-like) firing patterns are classically viewed as more reliable in terms of neurotransmission compared to regular firing (reviewed in Cooper 2002; Lisman 1997). This may imply that there is a constant increased release of ACh in Pitx3-deficient mice, which is regulated at the level of the ACh neurons themselves. Since irregular firing patterns in the mutant mice may alternatively have caused a more variable cholinergic tonus in the NAc of Pitx3-deficient mice compared to wildtype mice, we also determined the effects of spontaneous as well as evoked cholinergic modulation of sIPSC frequency. In doing so, we wanted to investigate whether a hyperdopaminergic input towards the NAc of Pitx3-deficient mice (Smits et al. 2005) may cause downstream changes in the microcircuitry, for instance via alterations in the expression and/or function of nAChRs in the NAc.

**Endogenous nicotinic modulation of GABAergic synapses.**

In wildtype and in some - but not all - of the Pitx3-deficient mice, the effects of mecamylamine were qualitatively similar to those observed in control rats, i.e. mecamylamine is likely to produce a consistent reduction in the amplitude of sIPSCs in individual recordings. As argued previously the nicotinic modulation of GABAergic input
onto medium spiny neurons is best explained by presynaptic activation of nAChRs on GABAergic interneurons. The endogenous activation of these nACh receptors, most likely at the level of the somata of the interneurons, would induce presynaptic firing (de Rover et al. 2002, 2004; Koos and Tepper 2002) leading to an upregulation of probability of GABA vesicles being released and therefore affect amplitude and/or frequency of the sIPSCs in the medium spiny neurons. Hence it may be concluded that nicotinic modulation of sIPSCs may occur both in wildtype and in Pitx3-deficient mice.

However, the effects of mecamylamine in mice were not as straightforward compared to those previously described for rat slices (de Rover et al. 2002, 2004), possibly due to the difference in size of the NAc of the animals used (age difference and difference between rats and mice). The smaller NAc used in the present research may have increased the risk that some of our recordings were made in the NAc core instead of the NAc shell as described in the Materials and Methods section. Further, the increase in sIPSC frequencies caused by the nicotinic receptor blocker mecamylamine that we found in some cells from both types of mice may be due to indirect effects upstream in the microcircuit.

**Evoked cholinergic modulation of GABAergic synapses**

In a final attempt to more precisely pinpoint the moment of ACh release and the extent to which nicotinic modulation of sIPSCs in the medium spiny neurons occurs, we manipulated the timing of spontaneous firing of ACh neurons in the paired recording experiments. In wildtype animals, we observed that this caused a consistent increase in sIPSC frequency, without any effect on the sIPSC amplitude. In contrast, in Pitx3-deficient mice no effects whatsoever were observed. Hence, we conclude that in Pitx3-deficient mice the cholinergic neuromodulation of inhibitory connectivity in the NAc is strongly affected.

This clear-cut result currently lacks the elucidation of underlying mechanism, however there are several possible explanations. One possible explanation is that the
dopaminergic tonus normally acts as a gating mechanism controlling cholinergic modulation of GABAergic inhibition in the NAc, similar to the role dopamine is considered to play in the prefrontal cortex (Braver et al., 1999; Dreher and Burnod, 2002). A hyperdopaminergic tonus has been reported to be present in the NAc of Pitx3-deficient mice (Gonon and Buda 1985; Kuhr et al. 1987; Smits et al. 2005; Suaud-Chagny et al. 1992). This may cause “abnormal gating” and thus apparent insensitivity of medium spiny neurons to changes in firing rate of ACh neurons. Alternatively, it is possible that in these mutant mice alterations in the excitation-secretion coupling in ACh neurons and/or desensitization of nAChRs may have occurred. Thus the Pitx3-deficient mice may have undergone developmental alterations of the NAc microcircuitry compared to wildtype mice.

**Functional implications**

Previously, both amphetamine and morphine pretreatment were shown to induce an increased mecamylamine effect on sIPSC amplitudes, explained by increased ACh release in the NAc (de Rover et al. 2004, 2005). In contrast, in Pitx3-deficient mice the mecamylamine effect on the sIPSC amplitudes was decreased rather than increased. Thus it seems that not only the firing pattern of cholinergic interneurons in the NAc of Pitx3-deficient mice is affected, but also more downstream parts of the microcircuit may be affected by the increased dopaminergic tonus in the NAc of these mice. It remains to be investigated why this effect of an increased dopaminergic tonus in the NAc was found in this study and not in drug-treated rats (de Rover et al. 2004, 2005). Possible explanations could be that the microcircuit is different in the control situation. The present study was done in mice, whereas the previous drug treatment studies were done in rats. Further, in the present study the developing NAc microcircuit was affected by an increased dopaminergic input, whereas in the drug-treated rats an existing NAc microcircuit was affected.
At the behavioural level the present results may have two interesting implications. First, the differently developed NAc microcircuitry may serve as a compensatory mechanism. In wildtype mice, injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to a selective loss of SNC dopaminergic neurons (Bradbury et al. 1986), which in turn gives rise to Parkinsonian animal behaviour (Fredriksson et al. 1990; Gerlach et al. 1991). Although Pitx3-deficient mice lack dopamine in their dorsal striata, they do not display a severe Parkinson-like phenotype (Smidt et al. 2004a; Smits et al. 2006). The developmental alterations of the NAc microcircuitry in Pitx3-deficient mice may be underlying this compensated behaviour. Therefore, understanding these adaptations may help to find better symptomatic treatments for Parkinson’s Disease.

The second implication at the behavioural level is that the changed NAc microcircuitry as measured here, may be a general effect, downstream of an increased dopaminergic transmission in the NAc, and therefore a so-called common denominator. We previously showed that the average firing frequency of cholinergic interneurons is not different between amphetamine pretreated rats and saline pretreated controls, but there is a more pronounced bimodality in the firing patterns of cholinergic interneurons of amphetamine pretreated rats (de Rover et al. 2004). The present results show a similar change in the firing of the cholinergic interneurons in Pitx3-deficient mice. Therefore, we currently consider the possibility that the more pronounced bimodality in the firing pattern of cholinergic interneurons after amphetamine pretreatment is not a substance specific but rather a secondary effect caused by the increased dopaminergic tonus in the NAc.
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REFERENCES


Braver TS, Barch DM, and Cohen JD. Cognition and control in schizophrenia: a computational model of dopamine and prefrontal function. *Biol Psychiatry* 46: 312-328, 1999


Gonon FG, and Buda MJ. Regulation of dopamine release by impulse flow and by autoreceptors as studied by in vivo voltammetry in the rat striatum. *Neuroscience* 14: 765-774, 1985.


LEGENDS

Figure 1

Is there an effect of Pitx3-deficiency on the firing pattern of ACh neurons? (A) Example traces from two recordings (A1 and A2) from two slices from different wildtype mice. (B) Example traces like in A, but recordings were made in slices from three different Pitx3-deficient mice (B1, B2 and B3). (C) Two example histograms of intervals between APs, obtained by discarding the first 30 seconds of the recording and binning the intervals of the first 300 APs starting from that time point. The intervals are plotted on a log scale (x-axis). The histograms are from two different example recordings made in slices from two different wildtype mice (C1 and C2). Single lognormal curves were fitted to both histograms. (D) Same as C, but the recordings were made in two different Pitx3-deficient mice and a double lognormal curve was fitted to one of the histograms (D1). (E) Overview of the results of all the fits done in both types of mice. On the x-axis are the results of the fitting procedure plotted on a log scale: either one (single lognormal function) or two (double lognormal function) average AP intervals (measured as tau values) with corresponding standard deviations, depending on the peak width. On the y-axis are the different recorded cells, for wildtype mice (upper part) n=7 and for Pitx3-deficient mice (lower part) n=6.

Figure 2

Is the endogenous nicotinic effect on sIPSCs affected in Pitx3-deficient mice? (A) Current traces from a wildtype control mouse, recorded (A1) under control conditions and further on in the same recording, (A2) in the presence of 1 µM mecamylamine. (B) Current traces from a Pitx3-deficient mouse recorded (B1) under control conditions and further on in the same recording (B2) in the presence of 1 µM mecamylamine. (C) Like panels A, but a different recording from a different control mouse with a different effect of mecamylamine. (D) Like
panels in B, but a different recording from a different Pitx3-deficient mouse with a different effect of mecamylamine. (E1) Average effect of 1 μM mecamylamine on sIPSC frequencies (grey bar) normalized to control conditions (white bar), calculated by averaging the results of the fits. The horizontal light grey bar represents the variation under control conditions (see Materials and Methods) n= 8. (E2) Average effect of 1 μM mecamylamine on sIPSC amplitudes (grey bar) normalized to control conditions (white bar), calculated by averaging the results of the fits. The horizontal light grey bar represents the variation under control conditions (see Materials and Methods) n= 8. (F) Like panels E, but for Pitx3-deficient mice n= 7.

Figure 3

Cholinergic modulation of GABAergic synapses is affected in Pitx3-deficient mice. (A) Example of simultaneously recorded voltage and current traces, recorded in a wildtype mouse respectively in a cholinergic interneuron in current clamp and a neighbouring GABAergic neuron in voltage clamp. (A1) Recorded ACh neurons were kept quiet by hyperpolarizing current injection and baseline sIPSCs were recorded. (A2) Further on in the same recording ACh neurons were allowed to fire at their endogenous frequency (no current injection) and sIPSCs were recorded during AP firing in the ACh neurons. (B) Example traces like in A, but this recording was made in a Pitx3-deficient mouse. In this particular case the ACh neuron is endogenously firing at a higher frequency than in the wildtype mouse shown in A, but overall the firing frequencies were not significantly different. (C) sIPSC frequencies in wildtype mice. (C1) Example recording in which the total sIPSC number per 5 sec time window is shown in time. The arrow represents the moment that the ACh neuron is allowed to fire APs. (C2) Average effect of AP firing in the ACh neuron on sIPSC frequencies (grey bar) normalized to control conditions (white bar), calculated by averaging the results of the fits. The horizontal light grey bar represents the variation under control conditions (see Materials
and Methods) n= 7. (D) Like panel C, but for Pitx3-deficient mice n= 7. (E) sIPSC amplitudes in wildtype mice. (E₁) Example recording in which the average sIPSC amplitude per 5 sec time window is shown in time. The arrow represents the moment that the ACh neuron is allowed to fire APs. (E₂) Average effect of AP firing in the ACh neuron on sIPSC amplitudes (grey bar) normalized to control conditions (white bar), calculated by averaging the results of the fits. The horizontal light grey bar represents the variation under control conditions (see Materials and Methods) n= 7. (F) Like panel E, but for Pitx3-deficient mice n= 7.

Table 1. Overview of the effect of mecamylamine on sIPSC frequency and amplitude in 8 different cells from wildtype mice and 7 different cells from Pitx3-deficient mice.

For each cell the sIPSC frequency and amplitude were normalized to 100% under control conditions. Mecfrq is the average sIPSC frequency in the presence of mecamylamine expressed as a percentage of the average sIPSC frequency (in the same cell) under control conditions. Mecamp is the average sIPSC amplitude in the presence of mecamylamine expressed as a percentage of the average sIPSC amplitude (in the same cell) under control conditions. KS is the result of Kolmogorov-Smirnov testing of the sIPSC frequencies or amplitudes in the presence of mecamylamine against the corresponding control sIPSC frequencies or amplitudes.
Figure 1
Figure 2
Figure 3

A1 Wildtype

B1 Pitx3-deficient

C1

D1

E1

F1

E2

F2

$10 \text{ pA/mV}$

500 ms

Number/sec (%)

0 50 100 150 200 250 300

Frequency (%)

0 25 50 75 100 125 150 175

Amplitude/sec (%)

0 50 100 150 200 250 300

Frequency (%)

0 25 50 75 100 125 150 175

Amplitude (%)

0 25 50 75 100 125 150 175

APs

APs

APs

APs

APs
Table 1. Overview of the effect of mecamylamine on sIPSC frequency and amplitude in 8 different cells from wildtype mice and 7 different cells from Pitx3-deficient mice.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Mecfrq</th>
<th>KS</th>
<th>Mecamp</th>
<th>KS</th>
<th>Mecfrq</th>
<th>KS</th>
<th>Mecamp</th>
<th>KS</th>
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<tr>
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<td>69.3</td>
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