Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome

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Exposure to the antiepileptic drug valproic acid (VPA) during gestation causes neurofunctional and anatomic deficits in later life. At present, there are little human data on how early neural development is affected by chemicals. We used human embryonic stem cells, differentiating to neuroectodermal precursors, as a model to investigate the modes of action of VPA. Microarray expression profiling, qPCR of specific marker genes, immunostaining and the expression of green fluorescent protein under the control of the promoter of the canonical neural precursor cell marker HES5 were used as readouts. Exposure to VPA resulted in distorted marker gene expression, characterized by a relative increase in NANOG and OCT4 and a reduction in PAX6. A similar response pattern was observed with trichostatin A, a potent and specific histone deacetylase inhibitor (HDACi), but not with several other toxicants. Differentiation markers were disturbed by prolonged, but not by acute treatment with HDACi, and the strongest disturbance of differentiation was observed by toxicant exposure during early neural fate decision. The increased acetylation of histones observed in the presence of HDACi may explain the up-regulation of some genes. However, to understand the down-regulation of PAX6 and the overall complex transcript changes, we examined further epigenetic markers. Alterations in the methylation of lysines 4 and 27 of histone H3 were detected in the promoter region of PAX6 and OCT4. The changes in these activating and silencing histone marks provide a more general mechanistic rational for the regulation of developmentally important genes at non-cytotoxic drug concentrations.

INTRODUCTION

Human pluripotent stem cells (PSC) and their progeny play an ever-increasing role as model systems to investigate toxicant effects and resultant human pathologies affecting the nervous system. Proof-of-concept for disease modeling with PSC has been provided by the use of induced PSC (iPS) from individuals with diseases caused by defined mutations (1) or from patients affected by complex neurodevelopmental and neurodegenerative diseases with unclear genetic background (2–4). Several pathologies related to disturbed neurogenesis, such as the fetal valproate syndrome (FVS), are triggered by chemicals or early-life stress in genetically normal individuals (5–9). To mimic features of such drug-induced human neurodevelopmental defects and to investigate the underlying mechanisms, new models are required. These combine in vitro neurodifferentiation of iPS from healthy donors or of human embryonic stem cells (hESC), with defined chemical exposure protocols.

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The FVS is triggered by the anti-epileptic drug valproic acid (VPA). It may manifest itself, for example, in spina bifida, cleft palate, autism–spectrum symptoms or lowered IQ scores in children exposed to the drug in utero (10,11). A characteristic feature of such chemical-induced developmental neurotoxicity (DNT) is that the sensitivity to the drug and the outcome of the treatment strongly depend on the time period of exposure (12,13). For instance, adverse neurodevelopmental effects of VPA are only seen after treatment at sharply defined time points of development, such as days 8–10 in mice (14–16). In humans and rodents, a major target of VPA is neural tube closure, which is the developmental time period when exposure is most detrimental (17–19). The effects of VPA on cortical organization and its triggering of autism also depend on a critical exposure period that coincides with the neural tube development (18).

An in vitro model of disturbed neural tube formation would need to examine generation of neural precursors from PSCs. A particularly synchronized and efficient neuroepithelial differentiation of hESC allows such mechanistic studies and is triggered under culture conditions that limit BMP4 and TGFβ family signaling by dual Smad inhibition (20). The resultant homogeneous population of neuroepithelial precursor (NEP) cells is characterized by the expression of PAX6, a marker found, for instance, in neural progenitor cells of the dorsal forebrain. The presence of this transcription factor marks the onset of neural tube closure in human embryos at 22–23 days of embryonic age (21). In addition, PAX6 is a target gene down-regulated in embryos exposed to VPA (22,23). NEP cells also express HES5, a target gene of Notch signaling (24), that is typically expressed in vivo in the neural tube. Thus, the investigation of the generation of NEP from hESC, under the influence of VPA and related toxicants, allows for the study of mechanisms relevant to early neurodevelopmental defects.

VPA has been reported to induce reactive oxygen species (25), to interfere with key neurochemical processes (26), and to affect WNT signaling as well as several other cellular processes (27–29). Alternatively, epigenetic effects resulting from the inhibition of histone deacetylases (HDACs) by VPA (30) may explain altered differentiation programs. The consequent increased acetylation of histones leads to chromatin opening and activation of gene transcription. This occurs within hours of exposure in stem cells, neural cells or in the neural tube of rodent embryos (31–34). Transcriptional and morphological effects of VPA in animals are reproduced by more specific HDAC inhibitors (HDACi). For instance, trichostatin A (TSA), which is more specific than VPA for HDACi, and at least 10,000-fold more potent with respect to enzyme inhibition (35), reproduces developmental effects of VPA in animals (36).

We initiated this study to model the disturbed early neural development triggered by HDACi in human cells. Using VPA, or the more specific tool compound TSA, we asked the question whether a set of marker genes that allows the characterization of drug-induced neurodevelopmental disturbances, such as those related to features of the FVS, can be identified. Fingerprint patterns of gene expression have been used previously to identify changes in neural cell populations exposed to environmental toxicants (12,37). In our study, it was particularly important to distinguish between the known short-term transcriptional activation caused by HDACi, and the still unknown long-term changes of the overall transcriptional program, triggered indirectly by disturbed neurodevelopment. We studied the requirement for prolonged drug exposure during a critical time period and wanted to find out whether changes were observable even after the drug was washed out for 1–2 days. Then, we asked whether a change of the histone code, e.g. by histone methylation, may link stable up- and down-regulations of neurodevelopmental gene expression to initial drug effects at the level of individual genes. Our study provides an explanation for the drug-induced deregulation of key neurodevelopmental transcription factors, such as PAX6, by examining trimethylation of H3K27 and H3K4 at their promoter.

RESULTS

Patterns of normal and disturbed neuroectoderm formation

A recently established protocol, based on dual SMAD inhibition, allows for the highly efficient and directed differentiation of PSCs to NEP cells (20). We used this procedure for the differentiation of hESC, and characterized their phenotypic changes over time. The process started with a homogeneous population of OCT4-positive cells, and after 6 days of differentiation (DoD) in adherent cultures, the cells stained positive for the NEP markers PAX6 and nestin (Fig. 1A). Scoring of the different cell types showed that on DoD6 ~90% of the cells were PAX6 positive and Nestin positive (Fig. 1B). For a more comprehensive characterization of the differentiation track, whole genome gene expression analysis was performed on hESC, as well as, DoD6 and DoD10 cells. Two-dimensional principal component analysis covering 41% of the total variance showed a clear separation of the three cell populations (Fig. 1C). The transcripts that were up-regulated on DoD6, or on DoD6 and DoD10, showed a highly significant statistical overrepresentation of several gene ontologies (GOs) related to neural differentiation and neurogenesis, but no indication of other differentiation processes. The genome-wide expression profiling, as a general overview, was complemented by detailed qPCR analysis of a set of differentiation markers. The hESC were differentiated for up to 10 days and mRNA was obtained at different time points. The NEP markers PAX6, FOXG1, OTX2 and SOX1 were up-regulated, whereas the stem cell markers NANOGEN and OCT4 were down-regulated. As most of the changes were pronounced (up to 100-fold) and mainly occurred from DoD0 to DoD6 (~90% of total effect size), this time window and these endpoints appeared most suitable to investigate disturbances in the differentiation (Fig. 1D).

To test the response dynamics and reproducibility of this experimental system, we performed some controlled manipulations and examined their effect on a profile of marker transcripts. First, the standard differentiation protocol was modified by omission of the BMP signal blockers Noggin and dorsomorphin. The time-dependent decrease in OCT4, and the increase in PAX6, SOX1 and OTX2 were less pronounced (Supplementary Material, Fig. S2A). This resulted
in a relative increase in OCT4 and a decrease in the NEP markers when compared with normal differentiation (Fig 1E). As expected, a very similar pattern of changes was observed when BMP4 was added during the normal differentiation protocol (Fig. 1F, Supplementary Material, Fig. S2B).
Under these conditions, increases in the endodermal marker $SOX7$ and the trophoectodermal marker $CDX2$ indicated a deviation of neuroectodermal differentiation to other cell specificities (Fig. 1F). As a second manipulation, either the inhibitor of the endogenous TGFβ receptor family signaling (SB-431542) was omitted during the differentiation, or an excess of the TGFβ receptor family ligand, activin A, was added. Under these conditions, the normal time-dependent changes of stem cell markers and NEP markers were strongly attenuated (Fig. 1G and H, Supplementary Material, Fig. S2). However, the expression of genes specific for endodermal ($SOX7$), mesodermal ($T$) or trophoectodermal ($CDX2$) lineages were not altered (data not shown). Altogether, these findings confirmed that our set of marker genes can indicate disturbed neuroectodermal differentiation and the kinetics of gene expression changes indicated that DoD6 is indeed a suitable time point for analysis. For this reason, VPA and other compounds were tested from DoD0–6 as the default condition (Fig. 2A).

**Disturbance of neuroectodermal differentiation by VPA**

DNT of VPA is of great clinical concern, but the drug’s effects on early neural specification are not well characterized in human cells. To address this issue, a concentration range of 250–1000 $\mu$M VPA was tested for effects on NEP differentiation (Fig. 2B). None of the investigated concentrations resulted in significant cytotoxicity (Supplementary Material, Fig. S3A). On DoD6, the mRNA levels of differentiation markers of treated cells and solvent controls were compared. The stem cell markers $OCT4$ and $NANOG$ were significantly higher in the presence of VPA concentrations $\geq 600 \mu$M, while $PAX6$ and $OTX2$ were greatly decreased. $SOX1$ and $FOXG1$ were not affected (Fig. 2B). Only concentrations of 1 $\mathrm{mm}$ resulted in the up-regulation of $CDX2$ and $T$ (data not shown).

In order to gain some insight into the specificity of the neurodevelopmental disturbance triggered by VPA, we compared its effect to those of other compounds (at known bioactive concentrations) with a potential to affect early neuroepithelial development. Neither the gamma-secretase inhibitor DAPT nor the recently discovered murine neurodevelopmental enhancer phenazopyridine (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers. In contrast, stimulation of WNT signaling by the glycosylphosphatidylinositol (38) or sonic hedgehog (SHH) affected the chosen set of markers.

Specific DNT of HDAC inhibitors

The effects of VPA were observed at clinically relevant concentrations, but this compound has low affinity to its multiple targets. Therefore, we used TSA, a highly potent HDACi, to investigate whether selective inhibition of histone deacetylation would reproduce the effects of VPA. First, cells were differentiated in the presence or absence of TSA for different periods of time. $OCT4$ was down-regulated less in the presence of TSA, and $PAX6$ did not increase as much (Fig. 3A). Also, $NANOG$ and $OTX2$ behaved the same way as they did when treated with VPA. $FOXG1$ and $SOX1$ showed complex biphasic responses (Supplementary Material, Fig. S5A). The concentration–response effects of TSA ($2.5–15 \mathrm{nm}$) were examined in more detail after treatment from DoD0–6. Increasing drug concentrations (significant effects at $\geq 7.5 \mathrm{nm}$) led to a strong decrease in $PAX6$ and $OTX2$, whereas $SOX1$ and $FOXG1$ were not affected (Fig. 3B). TSA concentrations of up to 10 $\mathrm{nm}$ did not result in a decrease in viability (Supplementary Material, Fig. S3B and C). Only concentrations of $\geq 15 \mathrm{nm}$ resulted in increases in other germ layer markers (data not shown). Thus, the response to TSA was remarkably similar to the one of VPA.

The mRNA markers only measured transcriptional changes averaged over a culture dish. We also used a NEP differentiation endpoint that can be assessed on the level of a single cell to gather information on the effects of HDACi on individual cells. $Hes5::GFP$ is a hESC cell line expressing GFP under the control of the $Hes5$ promoter, a Notch-target gene switched on in early neural precursor cells. These cells were analyzed by flow cytometry at different time points after initiation of NEP differentiation. A GFP-positive subpopulation comprising 50% of all cells was observed on DoD7 and at DoD10 ~75% of the cells expressed GFP (Fig. 3C). Due to the sufficient number of GFP-positive cells, we chose to perform all further analyses on DoD7. To assess the response dynamics of this endpoint, we initially tested the effect of the Notch signaling inhibitor DAPT. $Hes5::GFP$ cells, differentiated in the presence of DAPT from DoD0–7, showed decreased GFP expression. A significant reduction was also observed with treatment only from DoD6–7, as expected from the direct Notch-dependence of $HES5$ expression (Fig. 3D). In this system, we investigated the effects of HDACi. TSA concentrations $\geq 2.5 \mathrm{nm}$ and VPA concentrations $\geq 250 \mu$M led to a significant decrease in GFP-positive cells (Fig. 3E). These results confirmed the findings obtained with the marker genes. As a complementary approach, we chose to examine the effect of HDACi on the expression of the NEP marker $PAX6$ at the protein level. Immunocytochemical analysis of cells differentiated for 6 days showed that incubation with TSA or VPA during that time significantly reduced $PAX6$ staining (Fig. 3F). As TSA fully reproduced all the effects on NEP differentiation observed with VPA, this more specific drug was used for further characterization of the mode of action.

**Defining the window of sensitivity of NEP differentiation towards TSA**

In a differentiating tissue or under respective in vitro conditions, cells continuously change their phenotype. Therefore, also the targets and pathways that are affected by chemicals could be different when exposed at different stages of development. To gain more information on the processes affected by TSA, we applied the HDACi during different treatment windows and investigated the changes of $PAX6$ expression.
Treatment at the beginning of differentiation (DoD0–2) and analysis on DoD6 showed only minor disturbances caused by the chemical. Treatment only from DoD4–6 also did not affect the NEP marker. In contrast, prolonged treatment from DoD0–4 resulted in a maximum response on DoD6, even though the drug was absent during the last 2 days (Fig. 4B). Thus, the effects described for the exposure from DoD0–6 were probably not the direct result of transcriptional changes triggered by the presence of TSA, as described in short-term studies using different cell systems (31,39,40). They rather seemed to indicate altered NEP differentiation due to prolonged exposure during DoD0–4. A large-scale deviation from the normal differentiation trajectory was also observed when the overall transcriptome was examined after exposure to TSA. Principal component analysis (PCA) showed the emergence of an entirely different population under these conditions (Fig. 1C). Altogether, 2500 genes were differentially expressed. Among the 29 GOs most significantly overrepresented in the genes up-regulated in TSA-treated cells, 14 indicated development of non-neural tissues. All three GOs that were overrepresented among the down-regulated genes were related to neural development (data not shown). The genes down-regulated by TSA were compared with those that result in neural tube defects (NTD), when knocked-out in mice. This alignment resulted in 14 candidates that may be associated with human neurodevelopmental defects (Supplementary Material, Fig. S6).

Prolonged exposure outside the critical window of TSA susceptibility, for instance from DoD4–10, did not affect NEP differentiation, as assessed by measurement of OCT4 and PAX6 expression (Fig. 4C). The latter effect was examined in detail for a wide range of TSA concentrations (2.5–25 nM), and for further differentiation markers [FOXG1, SOX1, OTX2, NANOG (Supplementary Material, Fig. S5B)]. We also used Hes5::GFP cells to confirm, by an independent approach, that DOD0–4 was the critical window for HDACi effects on NEP differentiation. Exposure to TSA during DoD0–10 resulted in a pronounced down-regulation of GFP expression, indicative of disturbed NEP differentiation. In contrast, no effect was observed when the chemical was present during DoD4–10. The positive control, DAPT, reduced the GFP-positive cell population with either treatment. The negative controls SHH and phenazopyridin had no effect at any treatment period and omission of the BMP4 signaling inhibitors (noggin + dorsomorphin) at earlier time periods (DoD0–10) affected cell differentiation as expected, while late omission after fate decision (DoD4–10) had no effect on proper NEP formation (Fig. 4D). In summary, TSA

Figure 3. Disturbance of neuroectodermal differentiation by TSA. (A) Cells were exposed to TSA (10 nM) or solvent (control) from DoD0 to DoD8. Transcript levels were determined by qPCR and the ratio of TSA versus control (ctr) was calculated for each time point. Data are means ± SEM of three experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (B) Differentiating cells were exposed to TSA from DoD0–6. Marker gene transcript levels are displayed relative to untreated cells (DoD6 ctr). Data are means ± SEM of three to seven experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (versus DoD6 ctr). (C) Reporter cells (hESC expressing GFP under the Hes5 promoter) were differentiated for up to 10 days. The amount of GFP-positive cells was quantified by flow cytometry and normalized to the total number of cells. ***P < 0.001 versus DoD0. (D) Hes5 reporter cells were differentiated in the presence of DAPT (2.5 μM) for the indicated time periods. ***P < 0.001 versus ctr. (E) Hes5 reporter cells were differentiated in the presence of TSA or VPA from DoD0–7. GFP-positive cells were measured in three to nine experiments. *P < 0.01, ***P < 0.001 versus ctr. (F) hESC were differentiated in the presence or absence of 10 nM TSA or 0.6 mM VPA. At DoD6, cells were fixed and stained for PAX6. DNA was stained with H-33342. Scale bar: 50 μm.
affected the NEP differentiation program only after early and prolonged (>2 days) exposure.

Epigenetic modifications triggered by VPA and TSA

Although VPA has many known targets, it has been suspected for a long time that its activity as an HDACi is related to its developmental toxicity. The use of more specific and potent compounds, such as TSA, is necessary to further explore this hypothesis. We compared the two drugs with respect to their effect on histone modification. Both VPA and TSA increased H3 acetylation in differentiating cells rapidly (within 24 h) and to a similar degree, at the concentrations used for most endpoints in this study (10 nM TSA or 0.6 mM VPA) (Fig. 5A). This modification of the histone code is known to open the chromatin structure at the respective sites and to facilitate transcription. One explanation for decreased transcription may be the regulation of gene products that indirectly affect the promoter of down-regulated genes. To obtain some preliminary evidence for such a process, we examined a small, heterogeneous set of genes involved in different epigenetic processes. They involved TET2, and DNMT3B (DNA methylation), BMI1, CBX8 and PHC2 (polycomb complex proteins), HDAC9 and KAT2B (histone acetylation), NEK6 (chromosome segregation) and BAF60C (chromatin remodeling). Comparison of DoD6 cells differentiated in the presence or absence of TSA showed that e.g. PHC2, a representative of the polycomb group 1 complex, was up-regulated by the treatment (normal expression level of 1.5 ± 0.1 compared with hESC, increasing to 6.6 ± 1.7 in the presence of TSA). At the same time, genes affecting acetylation were significantly down-regulated (KAT2B, HDAC9). This may be indicative of secondary modifications of the histone code that contribute to the overall action of HDACi (Fig. 5B).

Such modifications were examined directly by chromatin immunoprecipitation (ChIP) for modified histones and their enrichment in the promoter of PAX6, OTX2 and OCT4 was quantified. H3K4 trimethylation (H3K4me3), as a mark for open chromatin, and H3K27 trimethylation (H3K27me3), as a mark for closed/silenced chromatin, were investigated in treated and untreated DoD6 cells. For many genes, the ratio of H3K4me3/H3K27me3 enrichment correlates with transcriptional activity (H3K4me3/H3K27me3) or silencing (H3K4me3, H3K27me3) (41–43). For the supposed housekeeping gene GAPDH, this ratio was 1 (active gene), and it was slightly further increased by TSA (Fig. 5C, Supplementary Material, Fig. S7). As expected, the ratio was < 1 on the OCT4 promoter (silenced) in control cells, and TSA led to a doubling of the ratio (trend towards less silencing). The PAX6 promoter was switched from an open to a silenced chromatin type in cells treated with TSA. This inversion of the ratio originated from a slight decrease in H3K4me3, together with a significant increase in H3K27me3 (Supplementary Material, Fig. S7). The promoter of OTX2 had higher K4me3 levels and lower H3K27me3 levels in untreated cells compared with TSA-treated cells (Fig. 5C, Supplementary Material, Fig. S7). All these findings correlated well with the observed changes in transcript levels (Fig. 3) and they confirmed our hypothesis that HDACi can affect key
transcriptional deregulation associated with disturbed neural differentiation that was compound-specific. The initial effects of HDAC inhibition were linked to the stably disturbed neural development by secondary epigenetic effects exemplified by altered methylation patterns of histones in the promoters of key transcription factors.

**Marker profile of normal and disturbed NEP differentiation**

We chose VPA for proof-of-concept experiments, as effects of this drug are well-documented in humans and animals. Then, we focused on TSA, to explore the role of HDACs as a specific target. Both compounds disturb very early neural development in animals (36) and several symptoms of FVS in humans are related to chemical effects during the most initial stages of nervous system formation (18,19). Our model system was based on the differentiation of hESC and the generated NEP correspond roughly to cells found at initial stages of neurulation (Fig.6A) (44). Differentiation markers used in this study were chosen, as they are well-established indicators of cellular patterning and regionalization of the neural plate (20,44). In particular, PAX6 is widely used as an early neural marker (45,46).

Correct formation and closure of the neural tube depend on a large number of genes, and over 50 candidates for NTD have been identified on the basis of mutant mouse models (47). Screening of human analogues of such genes in patients with NTD yielded rather ambiguous results (48,49). As an alternative approach, transcriptional changes associated with NTD have been investigated in murine fetuses exposed to environmental toxicants. Many of the altered transcripts in this model corresponded to the genes associated with NTD in gene-targeted mice (50). We used our human DNT model for a similar comparison of altered transcripts with NTD genes. Fourteen of the genes down-regulated by TSA are known from murine knock-out models to be associated with NTD (48,51). These candidates deserve further investigation in the future.

**Timing and concentration range of exposure**

To link changes in gene expression to defined molecular drug actions, we used TSA for mechanistic studies. This HDACi has less off-targets than VPA (52) and we found both compounds to show similar effects, i.e. both compounds generated a new cell population with a characteristic gene expression profile. Early and prolonged treatment with TSA, but not early and short exposure or late treatment (even for prolonged periods) led to disturbed neural differentiation (Fig. 6A). Notably, the changes in the chosen marker transcripts differed largely and distinctively from those triggered by MeHg, another compound known to interfere with neurodevelopment (47,53). They also differed from those triggered by activation of the WNT pathway, which would be another potential target of VPA. In this context, it is important to consider the concentrations used in this study. For instance, all compounds were used in a range in which they did not trigger cytotoxicity. Cell death was negligible (<10%) even with exposure to 800 nM MeHg, although such concentrations are lethal at
later stages of neurodevelopment (53). The VPA concentrations used here correlate well with human serum levels (ranging from 0.3 to 0.8 mM) (29,54) and with the 1 mM concentration used in other studies in the absence of cytotoxicity (31,40). As the fetal/maternal concentration ratio has been estimated to be 1.7 and the half-life of VPA in neonates is rather prolonged, the concentrations chosen here appear to be within the range expected in vivo (55). Also the concentrations of TSA (10 nM) chosen here are well within the range in which the compound does not trigger the death of stem cells and shows high specificity for HDACs as a target (31,56).

Direct versus indirect regulation of genes by HDACi

Several elegant studies have compared structurally diverse HDACi with other potential teratogens to identify signature genes triggered in neural/stem cells by short-term exposure (e.g. 6 h) (31,57). Such ‘HDAC genes’ were also confirmed upon short exposure to VPA in an embryonic stem cell test system (40). In contrast to this, we did not find any significant correlation of the transcript changes triggered by 6 days exposure to TSA with such genes. The pattern of changes we observed seems to be dominated by effects due to the generation of a different cell population because of altered differentiation signals. The direct effects of HDAC inhibition may be compensated after this continuous exposure by secondary regulations. Such differences between short- and long-term exposure are commonly observed in other signaling pathways, such as the triggering of epidermal growth factor (EGF) receptors. EGF, or serum only, lead to strong MAPK activation for short time periods, but not upon continuous activation (58,59). Treatment with TSA from DoD0–4, with a subsequent washout of the drug from DoD4–6, showed the same effect on NEP differentiation and marker transcripts as continuous exposure from DoD0–6. This further suggests that the differentiation changes triggered initially in this model appear to become stable and independent of the primary drug effect. Such an effect corresponds to the neurodevelopmental effects triggered in humans by short drug exposure during a critical phase of development.

Epigenetic cause for DNT effect

What links the initial HDACi-dependent changes to the stable change of gene expression seen later due to altered differentiation (Fig 6B)? Histone hyperacetylation, as observed shortly after drug exposure, may indeed be the initial trigger. However, additional changes are required, as this type of chromatin modification leads to chromatin opening at the respective sites and this epigenetic state facilitates gene transcription.
Instead, an altered methylation of histones may lead to chromatin opening or compaction, depending on the site of modification. For instance, chromatin opening due to HDACi may be enhanced and stabilized by increased H3K4 trimethylation (60), and VPA is known to change H3K4 levels in the neuro-epithelium of mice (61). While these studies only looked at global levels of chromatin marks in the genome, we added here specific information on the level of individual genes, and on the ratio of a major activating (H3K4me3) and inactivating (H3K27me3) chromatin modification on a given promoter site. Thus, we provide a rationale for stable down-regulation of genes which occur as a secondary consequence of treatment with HDACi. A relative increase in H3K27me3 at the promoter of the pro-neural transcription factor genes PAX6 and OTX2 accompanied their down-regulation.

A further indirect level of regulation may be the altered activity of genes that code for enzymes involved in the modification of the chromatin. Indeed, we detected changes, e.g. in the expression of PHC2 which is part of the polycomb complex, and in HDAC9 and KAT2B, the altered expression of which may be responsible for secondary changes in the histone code. Such regulations may be independent of the primary drug effects and rather be diagnostic for the altered differentiation track resulting in a different cell population (Fig. 6B). This has important implications for in vivo markers of NTD, as chemicals do not only change transcription directly, but stable indirect changes may be observed even after cessation of exposure due to changes in neurodevelopment.

Conclusion

Human PSC have been used here in a new approach to model drug-induced neurodevelopmental disease. The transcriptional effects of HDACi differed from those found in short-term studies, as they reflected altered neurodifferentiation due to secondary changes of the histone code for key transcription factors. In future, it will be interesting to apply a widened marker gene panel to investigate drug-induced effects on early human neural development. More detailed investigations of chromatin modifications at pivotal promoters will be required to derive general rules for the relationship of such mechanisms and chronic/developmental toxicity.

MATERIALS AND METHODS

Materials

Accutase was obtained from PAA (Pasching, Austria). Gel- atine, putrescine, selenium, progesterone, apotransferrin, glucose, insulin, VPA, TSA, methylmercury (II) chloride (MeHg), DAPT and phenazopyridine hydrochloride were purchased from Sigma (Steinheim, Germany). FGF basic, noggin and sonic hedgehog were obtained from R&D Systems (Minneaplos, MN, USA). Y-27632, SB-43154 and dorsomorphin dihydrochloride were from Tocris Bioscience (Bristol, UK). CHR98014 was purchased from Axon Medchem (Groningen, the Netherlands). MatrigelTM was from BD Biosciences (MA, USA). All culture reagents were from Gibco/Invitrogen (Darmstadt, Germany) unless otherwise specified.

Neuroepithelial differentiation

hESC (H9 from WiCells, Madison, WI, USA), and the H9-based Hes5 reporter cell line (Hes5::GFP) (24), kindly provided by Mark Tomishima and Lorenz Studer (Sloan Kettering, NY, USA), were differentiated as described earlier in detail (20). For details see Supplementary Methods.

Immunostaining and flow cytometry

For immunostaining, cells were fixed in 4% paraformaldehyde and 2% sucrose prior to permeabilization in 0.3% Triton X-100 in PBS. After blocking in PBS containing 5% bovine serum albumin and 0.1% Tween-20 for 1 h, primary antibodies (Supplementary Material, Fig. S1a) were incubated for 1 h, at room temperature (RT). After washing, secondary antibodies were applied for 30 min at RT. DNA was stained with Hoechst H-33342, and cover slips were mounted in FluorSave™ reagent (Calbiochem, Merck). For densely growing cells (DoD6 or DoD10), cells were detached, replated as monolayer and fixed 1 h later for immunostaining.

For analysis of the hESC expressing green fluorescent protein (GFP) under the control of the Hes5 promoter (hereafter called Hes5::GFP), the cells were differentiated for up to 10 days. Cells were detached by accutase treatment and resuspended in PBS containing 2% FCS. The amount of GFP-positive cells was quantified with an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) and data were processed with CFlow Plus (Accuri Cytometers).

Quantitative real-time PCR and microarray analysis

At indicated DoD, cells were lysed in TriFast™ (Peqlab, Germany) and total RNA was isolated according to the manufacturer’s guide and reverse transcribed (SuperScriptII, Invitrogen). Quantitative reverse-transcriptase real-time PCR (qPCR) for each transcript (Supplementary Material, Fig. S1b) was performed using EVAGreen® SsoFast™ mix on a BioRad Light Cycler (Biorad, Muenchen, Germany). For quantification, qPCR threshold cycles were normalized in a first step to housekeeping genes [tatabox binding protein (TBP) and ribosomal protein L13 (RPL13A)]. If not stated otherwise, the data of cells treated with chemicals were then expressed relative to transcript levels of untreated control cells which had been grown and differentiated for the same amount of time. For this normalization, the 2^(-Delta Delta C(T)) method was used (62). Affymetrix chip-based microarray analysis (Human Genome U133 plus 2.0 arrays) was performed as described in Supplementary Methods.

Western blot and ChIP

Western blot (63) and chromatin immunoprecipitation assays on native chromatin (N-ChIP) (64) were performed according to the established protocols. For details and adaptations, see Supplementary Methods.
Statistics and data mining
For statistical analysis of experiments in which only one condition of the differentiation was changed, paired \( t \)-tests were performed using log-transformed expression values. For multiple comparisons, one-way ANOVA analysis was performed with log-transformed expression values relative to hESC. One-way ANOVA was followed by a Dunnett’s post hoc test of comparisons relative to untreated cells. All data shown, and all statistics performed, refer to biological replicates (= independent experiments, each consisting of several technical replicates). Over-representation of GOs was tested with gprofiler (65). Highly overrepresented GOs were selected, if they belonged to the term domain ‘biological process’, contained \( >20 \) candidates in the list associated with the functional term which had a hierarchy level \( >4 \), and had a hypergeometric \( P \)-value smaller than \( 10^{-12} \).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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