Review Article

Brain and Neuroscience Advances

Brain and Neuroscience Advances
Volume 1: 1–5

DOI: 10.1177/2398212817728229
journals.sagepub.com/home/bna
SAGE

Dead or alive? The manipulation of neuronal ensembles and pathways by daunorubicin

Zafar Iqbal Bashir and Paul James Banks

Abstract

Some of the outstanding questions in neuroscience today are aimed at understanding the cellular and network mechanisms responsible for learned behaviours. Being able to identify and subsequently manipulate those specific neurones previously activated in a behavioural episode is key to this endeavour. A number of different methods have now been developed that enable this to be achieved. In this article, we highlight the Daun02–daunorubicin method of disrupting neuronal activity. Despite the fact that the Daun02–daunorubicin method has been used for a number of years and has been applied across a number of different experimental systems, the mechanism by which Daun02–daunorubicin disrupts neuronal activity is not clear. In this article, we summarise some of the advances that have been made by using this technology and we discuss potential mechanisms by which Daun02–daunorubicin disrupts neuronal function.

Keywords

Daun02, daunorubicin, neuronal ensembles, engrams, learning, neuronal circuits, neural activation, neuronal silencing, cell death

Received: 16 May 2017; accepted: 10 July 2017

Introduction

Daunorubicin

The Daun02–daunorubicin method is based on the anthracycline family of compounds (daunorubicin, doxorubicin and adriamycin, among others) that has long been used in anti-cancer treatment, especially of tumours in breast, bile ducts, oesophagus, liver and leukaemia. These compounds are known to have a large range of different cellular effects (ranging from inhibition of DNA synthesis to generation of free radicals), although it is not clear which of these lead to their effectiveness in cancer treatment (Gewirtz, 1999). In addition to effectiveness as a cancer therapy, it was discovered that daunorubicin can have devastating consequences for nervous system function, resulting from axonal and cerebral atrophy (Mortensen et al., 1992). The impact of these compounds on neuronal function is of clinical interest because of potential deleterious neurological side-effects of cancer treatment, but is also of scientific interest because of the potential use of daunorubicin to disrupt neuronal activity to examine cellular and circuit mechanisms of behaviour.

Targeting daunorubicin

To target anti-tumor anthracycline therapy to specific cells, the Daun02–daunorubicin method was developed (Farquhar et al., 2002). This method utilised the ability of β-galactosidase to catalyse conversion of the inactive prodrug Daun02 to active daunorubicin, allowing targeting of just those cells that produce β-galactosidase. Retroviral transduction of murine pancreatic tumour cell lines or human breast and prostate cell lines with LacZ resulted in production of β-galactosidase and conversion of Daun02 to daunorubicin, resulting in cell-targeted toxicity. This use of the conversion of the inactive Daun02 to active daunorubicin by β-galactosidase opened up the possibility of targeting daunorubicin not only to cancer cells but also to a range of other biological systems.

Behavioural utility of disrupting neuronal function by daunorubicin

Neuronal circuits that mediate addiction-related behaviours

The Daun02–daunorubicin method was developed by Bruce Hope and colleagues (Koya et al., 2009) who made a key methodological advance by utilising the *Fos*-LacZ transgenic rat in which activation of the *Fos* promotor induces transcription of

School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol, UK

Corresponding author:
Paul James Banks, School of Physiology, Pharmacology and Neuroscience, University of Bristol, Biomedical Sciences Building, Bristol BS8 1TD, UK.
Email: paul.banks@bristol.ac.uk

LacZ, in turn producing β-galactosidase enzyme in *Fos*-expressing neurones. *Fos* is an immediate early gene which is upregulated by neuronal activity (Herrera and Robertson, 1996; Tischmeyer and Grimm, 1999) and can therefore be used to produce β-galactosidase in only those cells that have been strongly active in a particular task; those cells can then in turn be selectively manipulated using Daun02. The authors developed this technique in order to manipulate neuronal ensembles or a minority of sparsely distributed sets of neurones activated during learned behaviours and/or by specific stimuli (Cruz et al., 2013).

Using this approach, Koya et al. (2009) investigated the requirement of nucleus accumbens neuronal ensembles in the context-specific sensitisation of cocaine-induced hyperlocomotor activity. This behaviour is dependent on specific learned associations between cocaine effects and the cocaine administration environment. They first demonstrated that approximately 2–3% of nucleus accumbens neurones expressed *Fos* during the expression of context-specific sensitisation. Subsequently, they demonstrated that intra-accumbens injections of Daun02 in *Fos*-LacZ rats applied during the expression of this behaviour disrupted its subsequent expression 2–3 days later. These findings established a critical role for accumbens neuronal ensembles in mediating the aforementioned drug-context associations. Since then, several researchers have used a similar Daun02-based approach to identify neuronal ensembles in motivationally relevant brain areas (e.g. prefrontal cortex and amygdala) that encode learned associations about the behavioural effects of other drugs of abuse such as alcohol (De Guglielmo et al., 2016; Pfarr et al., 2015), nicotine (Funk et al., 2016), heroin (Bossert et al., 2011; Fanous et al., 2013), methamphetamine (Caprioli et al., 2017), cocaine (Cruz et al., 2014) or food reward (Suto et al., 2016; Warren et al., 2016) and the environmental cues associated with their availability. Collectively, these studies demonstrate the utility of the Daun02 method to establish causal relationships between activated *Fos*-expressing neurones and learned behaviours.

Neural circuits of motor control

One of the major side-effects of dopamine replacement therapy in Parkinson's disease is L-DOPA-induced (L-3,4-dihydroxyphenylalanine) dyskinesia (LID). This dyskinesia is associated with increased *Fos* expression in human striatum (Tekumalla et al., 2001) and in animal models of LID (Andersson et al., 1999) and may rely on hyperactivity (Hutchinson et al., 1997) of striatal medium spiny neurones (MSNs). To examine whether LID relies on activation within MSNs, Daun02 was used as a means to silence activity within these cells (Engeln et al., 2016). In this case, FosB-LacZ lentivirus was injected into rat striatum to produce β-galactosidase. Subsequent infusion of Daun02 resulted in a significant reduction in abnormal involuntary movements that normally occur during LID. Furthermore, Daun02 was also found to be effective in countering LID in MPTP-lesioned L-DOPA-treated macaques (Engeln et al., 2016).

Neural circuits of spatial and temporal learning

The Daun02 method has also recently been used to enable selective deactivation of specific connections between brain regions to address the role of these connections in learning and memory (Barker et al., 2017). This was achieved using an equine infectious anemia virus-based VSV-G/rabies-G fusion envelope protein lentiviral vector expressing LacZ injected into the medial prefrontal cortex (mPFC) to enable retrograde transduction of cells projecting directly to mPFC. Injection of Daun02 into those areas, such as hippocampus, would be converted to daunorubicin by β-galactosidase only in those (hippocampal) neurones which project to mPFC. Using this approach, it was demonstrated (Barker et al., 2017) that two distinct pathways from CA1 to mPFC carry different mnemonic information; thus, silencing of the projection from dorsal hippocampus to mPFC selectively disrupted judgements of temporal order learning while silencing of projections from intermediate hippocampus-mPFC disrupted spatial learning.

Therefore, the use of targeted neuronal manipulation achieved by expression of B-gal using either *Fos* activation or viral transduction of specific synaptic pathways to allow conversion of Daun02 to daunorubicin has proved to be an extremely useful method for investigations of neurones involved in behaviour.

Mechanisms of neuronal deactivation by Daun02–daunorubicin

While it has been demonstrated that the Daun02 method is a highly selective tool for targeting specific neuronal ensembles, the utility of the system may be limited by its reversibility. We shall now discuss that some studies find the effects of Daun02 are reversible and may result from decreases in neuronal excitability (Barker et al., 2017; Engeln et al., 2016) while others find that Daun02 induces apoptotic cell death in LacZ expressing cells (Pfarr et al., 2015). The latter finding will limit the number of behavioural trials in which Daun02 may be a useful manipulation and in some cases may limit experimental cohorts to a 'one-shot' experiment. Alternatively, it is possible that in the future, non-reversible apoptotic cell death could be used advantageously in humans to block deleterious memories.

Cell death has been considered as one of the primary actions of daunorubicin in its function as a cancer treatment. However, the mechanism by which cell death occurs in cancer therapy is still a matter of some debate (Gewirtz, 1999). In this review, we will focus on what is known about the neuronal toxicity of daunorubicin action.

Apoptotic neuronal death

Inadvertent intrathecal administration of daunorubicin into a human patient resulted in cerebral and axonal atrophy, suggesting progressive and prolonged neuronal destruction (Mortensen et al., 1992). These effects may at least in part be due to retrograde transport as shown by doxorubicin injections in striatum leading to cell death in substantia nigra (Van der et al., 1985). In support of increased cell death, it was shown that daunorubicin resulted in reduced cell viability, as measured by in vitro cell toxicity assays (Farquhar et al., 2002). Primary cerebral cultures treated with doxorubicin underwent apoptosis that was dependent on metalloproteinase inhibition (Wetzel et al., 2003). IP administration of adriamycin to mice resulted in increases in protein oxidation and lipid peroxidation, suggestive of oxidative

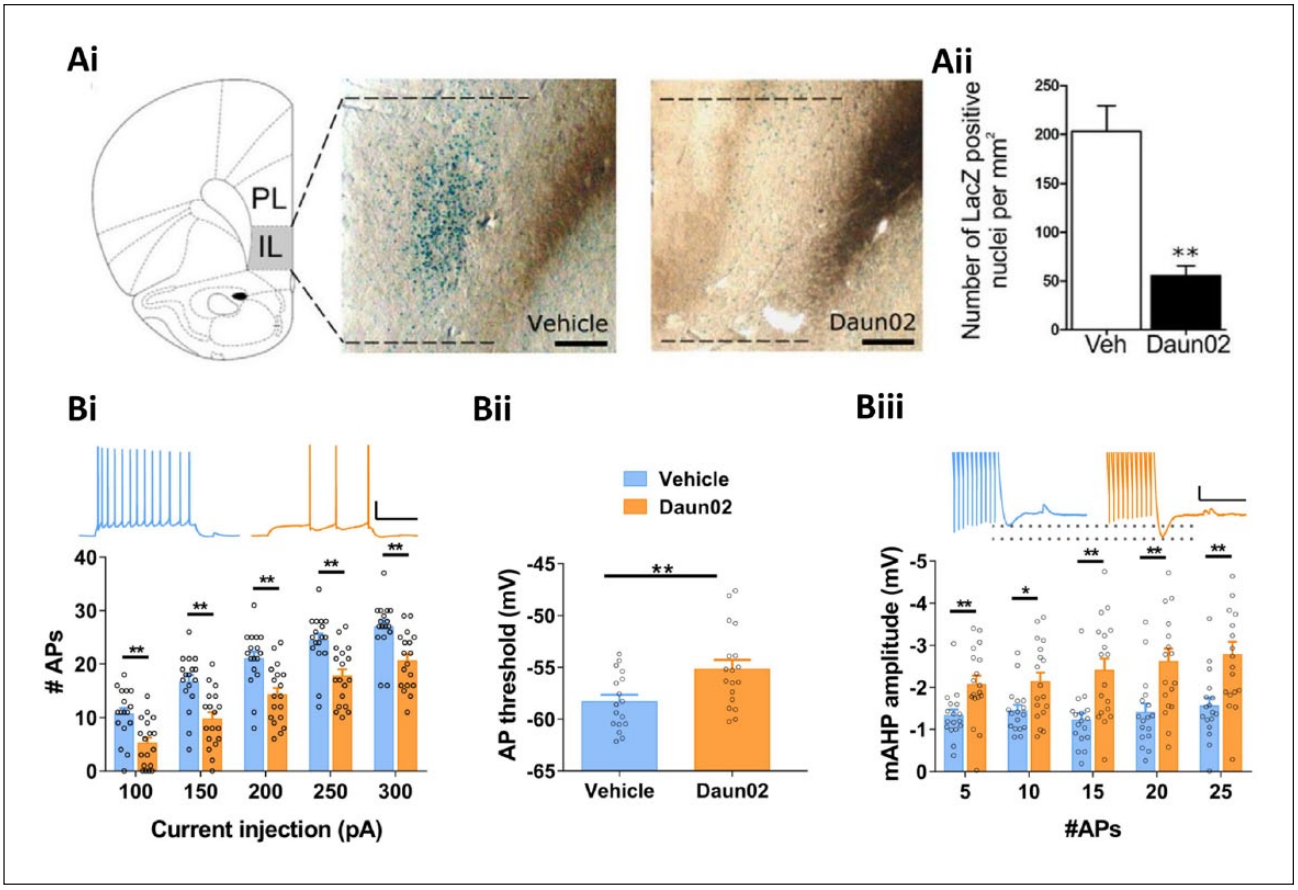

Figure 1. Contrasting effects of Daun02 on cellular condition. (A) cFos-LacZ transgenic rats were implanted with cannulae in infralimbic (IL) cortex after being trained to self-administer alcohol. Following cue-induced reinstatement, vehicle or Daun02 was infused into IL. (Ai) 3 days later, rats underwent a second reinstatement session and were sacrificed to undergo X-gal staining. (Aii) Daun02-treated animals show significantly lower levels of LacZ positive neurones compared to vehicle-treated animals; this is thought to be a result of cell loss caused by apoptosis. (B) Rats were given injections of a rabies-envelope pseudotyped virus encoding LacZ into prelimbic cortex to transduce retrogradely projecting neurones. Cannulae were implanted in hippocampal CA1 region and animals were infused with vehicle in one hemisphere and Daun02 in the opposite hemisphere 3 days before taking acute hippocampal slices: CA1 pyramidal cells from the Daun02 hemispheres fired significantly fewer action potentials in response to depolarising current than their vehicle counterparts (Bi), an effect thought to be due to an increase in the action potential threshold of those cells (Bii) and an increase in the magnitude of the afterhyperpolarising potential (Biii). Panel A adapted with permission from Pfarr et al. (2015) and panel B from Barker et al. (2017).

stress in the brain (Joshi et al., 2005). In primary neuronal cultures, daunorubicin was demonstrated to induce dose- and time-dependent cell toxicity as assessed by measures including lactate dehydrogenase release, MTT assay, Hoechst's staining to identify condensed chromatin, and caspase-3 activity. Interestingly, these effects were attenuated by memantine but not by other NMDA receptor antagonists (Jantas and Lason, 2009).

However, the above data do not provide evidence as to the mechanisms by which Daun02 may alter neuronal function in those studies of circuit function described above. Evidence for daunorubicin-induced apoptosis in behavioural studies was provided by injection of Daun02 into mPFC of pCAG-LacZ rats which constitutively express LacZ (see Figure 1(A)). This resulted in large levels of neurodegeneration, as assessed by fluorojade-B staining, which was prevented by co-infusion of the caspase inhibitor Z-VAD-FMK (Pfarr et al., 2015). Therefore, there is a good deal of evidence from cell lines, neuronal cultures, in vivo animal and human data that the anthracillin family of compounds can result in neuronal cell death.

Effects on neuronal activity

Early evidence from Santone et al. (1986) in a murine neuroblastoma cell line suggested that the daunorubicin family of compounds can have subtle effects on neuronal activity. Differentiation of the NIE-115 neuroblastoma cell line results in these developing sodium- and calcium-dependent action potentials. In the presence of tetrodotoxin, to block Na channels, it was shown that either doxorubicin or daunomycin dose-dependently reduced action potential firing (Santone et al., 1986). Doxorubicin was shown in both aplysia sensory neurones and rat cortical cultures to produce an increase in ERK, p38 MAPK and CREB activation without any apparent changes in toxicity (assessed by cell shrinkage, membrane rupture and neurite degeneration). Interestingly,

doxorubicin was able to prevent long-term facilitation induced by 5-HT in aplysia neurones without having any effect on basal synaptic transmission or membrane potential by itself, suggesting that while there were no toxic effects, doxorubicin can impact synaptic mechanisms (Liu et al., 2014).

To determine the actions of daunorubicin on striatal MSNs, Engeln et al. (2016) showed in rat MSN primary cultures expressing LacZ that there was strong attenuation of action potential firing in response to depolarising steps following perfusion of Daun02. Importantly, the attenuation of excitability was reversible upon Daun02 washout, suggesting that any effects of daunorubicin are most likely due to reversible effects on the membrane or on specific ion channels. In addition, they were also able to demonstrate in brain slices that daunorubicin applied into the extracellular perfusate resulted in a similar attenuation of MSN excitability. Most recently, Barker et al. (2017) investigated effects of daunorubicin on neuronal function in rat hippocampal CA1 neurones. This was achieved by bilateral expression of LacZ in hippocampal CA1 region. Subsequently, Daun02 was infused into CA1 in one hemisphere and saline into the other hemisphere. Horizontal hippocampal slices were taken 3 days later and CA1 pyramidal neurone membrane properties assessed. These data showed that in the Daun02 hemisphere compared to control hemisphere, there was a decrease in action potential number, which can be explained by an increase in action potential threshold and an increase in the size of the afterhyperpolarisation; input resistance was not affected by Daun02 (see Figure 1(B)).

The studies of Engeln et al. (2016) and Barker et al. (2017) show similar results in that the number of action potentials was reduced by Daun02–daunorubicin treatment, but the underlying mechanism of this is not clear. The increased action potential threshold observed by Barker et al. (2017) may be explained by inhibition of voltage-gated sodium or calcium channels as observed previously (Santone et al., 1986). Additionally, the increased afterhyperpolarisation amplitude may be mediated by a potentiation of M- and/or h-currents (Gu et al., 2005). It is not clear whether the reduced firing observed by Barker et al. (2017) and Engeln et al. (2016) are mediated by the same mechanisms. The methodology and the time course of treatment differ between the two studies: Engeln et al. (2016) observe reduction in firing following acute daunorubicin application to a slice or after incubating LacZ-expression neuronal cultures in Daun02 for 2 h. In contrast, Barker et al. (2017) infused Daun02 into the hippocampus 3 days before assessing neuronal properties in vitro. As an infusate would not be expected to be active beyond a few hours (Day et al., 2003), this suggests that the conversion of Daun02 to daunorubicin in vivo has a lasting, possibly transcriptional, effect on those cells which express β-galactosidase. Together, the results of these studies imply that use of the terms 'silencing' and 'inactivating' neural ensembles may be inappropriate as the effect of Daun02 is merely a reduction in activity where neurones can still fire action potentials but with reduced number or frequency.

Cell death versus disruption of activity

There is strong evidence for the Daun02–daunorubicin method affecting neuronal function by either reducing cell activity or by inducing cell death. It is also possible that both effects of reduced excitability and cell death are occurring in the experiments of Barker et al. (2017) and Engeln et al. (2016), and that the electrophysiological recordings are necessarily selective for the former neurones. However, in the case of the behavioural experiments carried out by Barker et al. (2017), a single cohort of rats was used to perform the Daun02 experiments and these animals received multiple Daun02 infusions and yet were continually able to perform hippocampal-mPFC-dependent tasks at a high level when subsequently pseudo-randomly assigned to the vehicle condition. Given the strong expression of LacZ expression observed in the hippocampus in those animals, it appears unlikely that widespread cell death was occurring following Daun02 administration.

An alternative scenario to explain the differences observed in cell death and effects on neuronal activity may lie in the methods of expressing the LacZ gene: both studies which show non-neurotoxic, reversible effects of Daun02 (Barker et al., 2017; Engeln et al., 2016) use a viral strategy to transduce neurones with the LacZ gene, whereas Daun02 infusion in cFos-LacZ or pCAG-LacZ transgenic rats seems to result in cell death (Pfarr et al., 2015). If the expression method dictates the levels of β-galactosidase in labelled cells, this may dictate the rate of conversion of Daun02, potentially resulting in different maximal concentrations of daunorubicin. It remains to be seen under these in vivo conditions whether high concentrations are cytotoxic while lower concentrations merely affect cell excitability.

Conclusion

The use of Daun02–daunorubicin to probe causal relationships between neuronal activity or circuit function and behaviour has proved to be highly effective. However, under different conditions, daunorubicin may activate different mechanisms to reduce excitability or induce apoptosis. Therefore, different strategies may be appropriate for different experimental scenarios; thus, if the effects of deactivation in a range of paradigms are required, then a reversible effect, such as that produced by viral expression of the LacZ gene, would be the method of choice.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

The authors are supported by BBSRC funding (BB/L001896/1).

References

Andersson M, Hilbertson A and Cenci MA (1999) Striatal FosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. *Neurobiology of Disease* 6(6): 461–474.

Barker GR, Banks PJ, Scott H, et al. (2017) Separate elements of episodic memory subserved by distinct hippocampal-prefrontal connections. *Nature Neuroscience* 20(2): 242–250.

Bossert JM, Stern AL, Theberge FR, et al. (2011) Ventral medial prefrontal cortex neuronal ensembles mediate context-induced relapse to heroin. *Nature Neuroscience* 14(4): 420–422.

Caprioli D, Venniro M, Zhang M, et al. (2017) Role of dorsomedial striatum neuronal ensembles in incubation of methamphetamine craving after voluntary abstinence. *Journal of Neuroscience* 37(4): 1014–1027.

Cruz FC, Babin KR, Leao RM, et al. (2014) Role of nucleus accumbens shell neuronal ensembles in context-induced reinstatement of cocaine-seeking. *Journal of Neuroscience* 34(22): 7437–7446.

Cruz FC, Koya E, Guez-barber DH, et al. (2013) New technologies for examining the role of neuronal ensembles in drug addiction and fear. *Nature Reviews Neuroscience* 14(11): 743–754.

Day M, Langston R and Morris R (2003) Glutamate-receptor-mediated encoding and retrieval of paired-associate learning. *Nature* 424(6945): 205–209.

De Guglielmo G, Crawford E, Kim S, et al. (2016) Recruitment of a neuronal ensemble in the central nucleus of the amygdala is required for alcohol dependence. *Journal of Neuroscience* 36(36): 9446–9453.

Engeln M, Bastide MF, TOulme E, et al. (2016) Selective inactivation of striatal FosB/DeltaFosB-expressing neurons alleviates L-DOPA-induced dyskinesia. *Biological Psychiatry* 79(5): 354–361.

Fanous S, Guez-Barber DH, Goldart EM, et al. (2013) Unique gene alterations are induced in FACS-purified Fos-positive neurons activated during cue-induced relapse to heroin seeking. *Journal of Neurochemistry* 124(1): 100–108.

Farquhar D, Pan BF, Sakurai M, et al. (2002) Suicide gene therapy using E. coli beta-galactosidase. *Cancer Chemotherapy and Pharmacology* 50(1): 65–70.

Funk D, Coen K, Tamadon S, et al. (2016) Role of central amygdala neuronal ensembles in incubation of nicotine craving. *Journal of Neuroscience* 36(33): 8612–8623.

Gewirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochemical Pharmacology* 57(7): 727–741.

Gu N, Vervaeke K, Hu H, et al. (2005) Kv7/KCNQ/M and HCN/h, but not KCa2/SK channels, contribute to the somatic medium after-hyperpolarization and excitability control in CA1 hippocampal pyramidal cells. *Journal of Physiology* 566(3): 689–715.

Herrera DG and Robertson HA (1996) Activation of *c-fos* in the brain. *Progress in Neurobiology* 50(2–3): 83–107.

Hutchinson WD, Levy R, Dostrovsky JO, et al. (1997) Effects of apomorphine on globus pallidus neurons in parkinsonian patients. *Annals of Neurology* 42(5): 767–775.

Jantas D and Lason W (2009) Protective effect of memantine against Doxorubicin toxicity in primary neuronal cell cultures: Influence a development stage. *Neurotoxicity Research* 15(1): 24–37.

Joshi G, Sultana R, Tangpong J, et al. (2005) Free radical mediated oxidative stress and toxic side effects in brain induced by the anti cancer drug adriamycin: Insight into chemobrain. *Free Radical Research* 39(11): 1147–1154.

Koya E, Golden SA, Harvey BK, et al. (2009) Targeted disruption of cocaine-activated nucleus accumbens neurons prevents context-specific sensitization. *Nature Neuroscience* 12(8): 1069–1073.

Liu RY, Zhang Y, Coughlin BL, et al. (2014) Doxorubicin attenuates serotonin-induced long-term synaptic facilitation by phosphorylation of p38 mitogen-activated protein kinase. *Journal of Neuroscience* 34(40): 13289–13300.

Mortensen ME, Cecalupo AJ, Lo WD, et al. (1992) Inadvertent intrathecal injection of daunorubicin with fatal outcome. *Medical and Pediatric Oncology* 20(3): 249–253.

Pfarr S, Meinhardt MW, Klee ML, et al. (2015) Losing control: Excessive alcohol seeking after selective inactivation of cue-responsive neurons in the infralimbic cortex. *Journal of Neuroscience* 35(30): 10750–10761.

Santone KS, Oakes SG, Taylor SR, et al. (1986) Anthracycline-induced inhibition of a calcium action potential in differentiated murine neuroblastoma cells. *Cancer Research* 46(6): 2659–2664.

Suto N, Laque A, De Ness GL, et al. (2016) Distinct memory engrams in the infralimbic cortex of rats control opposing environmental actions on a learned behavior. *eLIFE* 5: e21920.

Tekumalla PK, Calon F, Rahman Z, et al. (2001) Elevated levels of DeltaFosB and RGS9 in striatum in Parkinson's disease. *Biological Psychiatry* 50(10): 813–816.

Tischmeyer W and Grimm R (1999) Activation of immediate early genes and memory formation. *Cellular and Molecular Life Sciences* 55(4): 564–574.

Van der Kooy D, Zito KA and Roberts DCS (1985) Evidence on the retrograde neurotoxicity of doxorubicin. *Neuroscience Letters* 53(2): 215–219.

Warren BL, Mendoza MP, Cruz FC, et al. (2016) Distinct Fos-expressing neuronal ensembles in the ventromedial prefrontal cortex mediate food reward and extinction memories. *Journal of Neuroscience* 36(25): 6691–6703.

Wetzel M, Rosenberg GA and Cunningham LA (2003) Tissue inhibitor of metalloproteinases-3 and matrix metalloproteinase-3 regulate neuronal sensitivity to doxorubicin-induced apoptosis. *European Journal of Neuroscience* 18(5): 1050–1060.